STRUCTURAL CHARACTERIZATION AND IMMUNOLOGICAL STUDIES OF LIPOPHOSPHOGLYCAN OF *LEISHMANIA DONOVANI*

THESIS

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BY

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I certify that the work presented in the following pages has been carried out by **Mr. Vahab Ali,** under my supervision and that in my opinion it is suitable for the award of **Ph.D.** degree in Biochemistry of the Aligarh Muslim University, Aligarh.

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THESIS

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This is to certify that the work embodied in this thesis entitled " STRUCTURAL CHARACTERIZATION AND IMMUNOLOGICAL STUDIES OF LIPOPHOSPHOGLYCAN OF *LEISHMANIA DONOVANI*" has been carried out by Mr. Vahab Ali, under my supervision. He has fulfilled the requirements for the degree of Doctor of Philosophy of Aligarh Muslim University, Aligarh regarding the nature and prescribed period of investigation of work. The work included in this thesis has not been submitted for any other degree and unless otherwise stated, is all original.

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TO

LOVING

PAPA AND MUMMY

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PREFACE

The protozoans are the most diverse and amongst the most ancient group of organisms in the eukaryotic kingdom. Many of their members are parasitic like, those belonging to the family, Trypanosomatidae (African trypanosomes, trypanosoma cruzi, *Leishmania* spp.) and the genera *Plasmodium Eimeria, babesia, Theileria, Toxoplasma* and *Entamoeba* are the cause of important diseases in humans and their domestic liverstock. Parasitic infections are a major health problem World wide especially in under developed and developing countries. In the recent past WHO has selected six important diseases in tropical and subtropical regions, in which human leishmaniasis is second in importance only to malaria, as it possesses much greater problem both in treatment and control. Several chemotherapeutic agents/drugs against human leishmaniasis are available but they suffer from serious limitations like toxic side effect and their mechanism of action poorly understood. Recent studies have shown that immunotherapy/immuno chemotherapy is much more promising than chemotherapy.

Recent and past experiences have indicated that cell surface molecules in view of their importance in interaction with the immune system, parasite recognition and uptake and survival in macrophages held a great promise as immunoprophylactic/vaccine candidate. LPG is a major cell surface glycoconjugate of *Leishmania* parasite and is thought to play an important role in the biology of the parasite.

We have isolated the LPG from promastigotes of UR6 strain, purified, characterized and studied its *in vitro* potential in providing protection against *L. donovani* promastigote infection in peritoneal macrophages. Furthermore, *in vivo* efficacy of LPG as a immunoprophylactic agent against *L. donovani* infection in Syrian golden hamsters has also been studied.

ABSTRACT

Lipophosphoglycan (LPG) is a major cell surface glycoconjugate of Leishmania parasites. This unusual glycoconjugate is present throughout the various phases of growth in Leishmania parasites. The promastigotes plasma membrane contains about 1.25 million copies/cell of LPG, accounting for atleast 25% of its cell wall. Structurally, LPG is composed of a neutral cap and a variable composition of a repeating phosphorylated disaccharide units attached via a conserved phosphosaccharide core to a phosphatidylinositol (PI) lipid anchor. All LPG molecules contain multiple unit of a backbone structure of PO_4-6-Gal(β 1->4)Man α 1-. These phosphorylated disaccharide repeat units are attached by α- glycosidic linkage. The disaccharide repeat units of L. donovani LPG has no substitutions in their backbone sequence whereas repeating units of L. mexicana LPG has approximately 30% of its galactose residues substituted at the C 3 hydroxyl with glucose (Glc) residues. The repeating units of the L. major LPG are the most complex, as more than 85% of the galactose residues are further substituted with small saccharide chains containing one to four residues of galactose, glucose, or the pentose arabinose. It has been demonstrated that the number of repeating units per LPG molecule directly depends on the growth stage of promastigotes. The average number of repeat units per molecule reported for different species are : 16 for L. donovani, 20 for L. mexicana and 14 for procyclic, 30 for metacyclic, 36 for amastigotes of L.

major. The overall structure of LPGs isolated from L. donovani, L. major, and L. mexicana promastigotes are similar, with a linear arrangement of caps, repeating units, glycosylated core and lyso-alkylglycerol lipid moiety. The most striking differences in LPG structure between the Leishmania species lies in the phosphorylated oligosaccharide repeats.

LPG plays an important role in the biology of the parasite due to its surface location, its developmental regulation during the life cycle and the reduce virulence of the LPG-deficient organisms. A variety of functions and activities of LPG in the mammalian host have been experimentally demonstrated or suggested. These include, involvelment in attachment and entry of promastigotes into mammalian macrophages, protection of parasite within phagolysosomal compartment and as a recognition molecule for the T-lymphocyte dependent immune responses characteristic of leishmaniasis. LPG is an inhibitor of protein kinase C, inhibitor of oxidative burst, inhibitor of viral fusion, signal transduction and scavenger of oxygen free radicals. Lipophosphoglycan has also been proposed to induce a protective immune response in mice and therefore, it is considered to be a candidate vaccine against leishmaniasis.

In recent years, the promastigote cell surface has received considerable attention in view of its importance in interaction with the immune system and for parasite recognition, uptake and survival in macrophages. The cell surface glycoconjugate, lipophosphoglycan (LPG) has been isolated, purified and characterized from Leishmania parasites of different species.

However, no attempt has been made to study the LPG from non-pathogenic strains. Hence, LPG from promastigotes of non-pathogenic strain UR6 was isolated, purified, characterized and its potential as immunoprophylactic agent and/or candidate vaccine against experimental visceral leishmaniasis was evaluated. Isolation of LPG was carried out using different organic solvent mixtures. Crude LPG was purified by size-exclusion, and hydrophobic affinity chromatography. The elution profile of LPG from Sephadex G-200 is indicating that LPG fractions eluted just after void volume, suggesting that it is a high molecular weight molecule. Single broad peak was obtained indicating the heterogeneous nature of LPG. The desaltation of LPG molecule which is eluted from Sephadex G-200 was necessary for removal of unwanted salts like, EDTA, NH_4^+, Mg^{++} etc. These salts were found to interfere in further purification and characterization studies. LPG purified by conventional methods failed to remove LPG-associated protein contaminants as they were tightly bound to LPG. These associated proteins were removed by the treatment with proteinase K enzyme and protein/peptide free LPG was obtained after purification from octyl Sepharose column. From 25.0 ml (27.0 gm) packed cells, about 150 mg of crude LPG was obtained. This on partial purification yielded 31 mg of partially purified LPG which on octyl Sepharose CL-4B column gave a yield of about 15.0 mg of pure LPG. Characterization of purified LPG was carried out by thin layer chromatography (TLC), SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and NMR

spectroscopy. The mobility of purified LPG on silica gel G coated plate in the solvent mixture 1-propanol/pyridine/H_2O (1:1:1, v/v) is shown a single diffuse spot. The R_f value of LPG in this solvent system was found to be about 0.66 ± 0.02, which is close to 0.62 reported earlier for the L. donovani promastigotes LPG of pathogenic strain. The purity of LPG molecule was assessed by SDS-PAGE followed by periodic acid schiffs (PAS) staining. On PAS staining of gel, LPG was the only carbohydrate staining species observed as it migrated as a single diffused band. The molecular weight of UR6 LPG obtained by the migration of standard proteins on SDS-PAGE, was found to be in the range of 20-25 kDa. One dimensional ^1H - NMR and two dimensional COSY NMR studies were carried out for structural characterization of the LPG molecule. The signal at δ 5.52 ppm was unambiguously assined to H-1 of mannose and connectivities could be traced out as cross peak to the H-2 (δ 3.71 ppm) and H-3 (δ 3.53 ppm) respectively. Similarly, the signal at δ 4.47 ppm can be unambiguously assinged to H-1 proton of galactose and connectivities could be traced out for H-2 (δ3.54 ppm) and H-3 (δ3.83 ppm) respectively. Further, connectivities could not traced out due to the in phase properties of diagonal peak. Hence, it is suggested that LPG molecule is obtained after final purification was pure. The LPG samples are devoid of any protein contaminants which was supported by the NMR spectroscopy as no signal of any $-NH_2$ was found during NMR studies except carbohyrates corresponding peaks

were observed.

The protective potential of L. donovani LPG, against L. donovani infection in peritoneal macrophages has been studied. The studies demonstrate that UR6 LPG inhibits binding of promastigotes of Dd8 strain to peritoneal macrophages in a concentration dependent manner. For 10 ng/ml LPG about 50% inhibition of attachment to peritoneal macrophages was obse rved. This inhibitory activity was enhanced from 60% to 78%, when the LPG concentration was increased from 50 ng/ml to 100 ng/ml, respectively. A maximum inhibition of about 85-90% was observed for 1000 ng/ml of LPG. No further enhancement in inhibition with increase in LPG concentration upto 2000 ng/ml was observed. Similar results were also observed when LPG was incorporated into small unilamellar vesicle (SUV) containing cholesterol. Furthermore, UR6 LPG provided a significant protection (about 85-90%) in peritoneal macrophages against promastigotes infection. This study is also supported by previous study (Kelleher et al., 1995) that LPG was unable to completely block attachment of promastigotes to macrophages, suggesting the involvement of other ligands supporting the earlier observation that the domain of LPG which is involved in binding of promastigotes to the macrophages, is conserved in the promastigotes LPG of both the Dd8 and UR6 strains of L. donovani.

In order to look into the possibility whether LPG mediated protection in macrophages system against L. donovani was either due to inhibition of attachment of promastigotes to macrophages

or killing of amastigotes inside the macrophages, studies with pre and post treatment of macrophages with UR6 LPG were carried out. It was observed that the peritoneal macrophages which were treated with LPG before being challenged with promastigotes provided significant protection against L. donovani infection. However, those macrophages which were first challenged with promastigotes and then treated with LPG provided similar pattern of infection as observed that for control. These observations, suggest that pretreatment of peritoneal macrophages is necessary for achieving protection which depends on the inhibition of attachment of promastigotes to peritoneal macrophages.

In vivo efficacy of LPG molecules against visceral leishmaniasis was evaluated in susceptible golden hamsters using LPG alone or incorporated in liposomes. Pretreatment of hamsters with LPG anywhere between 10-28 days prior to infection did provide significant protection against L. donovani infection. The group of animals receiving LPG alone (10 µg/animal) showed a relatively low protection of about 22-25% . The protective efficacy of LPG was enhanced significantly on its incorporation in liposomes. LPG incorporated in cholesterol containing multilamellar liposomses provided 40 percent protection whereas LPG incorporated in small unilamellar vesicles (SUV) provided about 65-70 percent protection. This protection was more than double as compared to LPG alone. Furthermore, for incorporation of LPG in negatively charged liposomes as similar percent

protection as that for neutral liposomes was observed. Studies on concentration dependence of liposomised LPG in protecting hamster against L. donovani infection showed that a single dose of 20 µg/animal of liposomised LPG incorporated in SUV provided a maximum protection of about 68 percent. The effectivenss of liposome incorporated LPG compared to LPG alone in protection against L. donovani in hamsters can be due to induction of an effective T-cell response. This possiblity is indicated from the fact that increased percent protection was observed on increasing the pretreatment schedule with liposomised LPG from 2 days to 21 days.

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(VAHAB ALI)

ABBREVIATIONS

LPG	:	LIPOPHOSPHOGLYCAN
GAL	:	GALACTOSE
MAN	:	MANNOSE
ARA	:	ARABINOSE
GLC	:	GLUCOSE
BSA	:	BOVINE SERUM ALBUMIN
GLC N	:	UNACETYLATED GLUCOSAMINE
CR-1	:	COMPLEMENT RECEPTOR-1
CR-3	:	COMPLEMENT RECEPTOR-3
MFR	:	MANNOSE FUCOSE RECEPTOR
FnR	:	FIBRONECTIN RECEPTOR
TNF	:	TUMOR NECROSIS FACTOR
PBS	:	PHOSPHATE BUFFER SALINE
EDTA	:	ETHYLENE DIAMINE TETRA ACETIC ACID
FCS	:	FETAL CALF SERUM
TLC	:	THIN LAYER CHROMATOGRAPHY
SDS-PAGE	:	SODIUM DODECYLSULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS
NMR	:	NUCLEAR MAGNETIC RESONANCE
kDa	:	KILODALTON
Spp.	:	SPECIES
gm	:	GRAM
mg	:	MILLIGRAM
ug	:	MICROGRAM
ng	:	NANOGRAM
M	:	MOLAR
mM	:	MILLIMOLAR
cm	:	CENTIMETER
mm	:	MILLIMETER
um	:	MICROMETER
nm	:	NANOMETER
ml	:	MILLILITER
ul	:	MICROLITER
mA	:	MILLIAMPERE
A^{o}	:	ANGSTROM

^{o}C	:	DEGREE CENTIGRADE
O.D.	:	OPTICAL DENSITY
MIN	:	MINUTE
ppm	:	PARTS PER MILLION
T_H1	:	T HELPER CELL 1
T_H2	:	T HELPER CELL 2
IL	:	INTERLEUKIN
IFN	:	INTERFERON
PKDL	:	POST KALA-AZAR DERMAL LEISHMANIASIS
VL	:	VISCERAL LEISHMANIASIS
CL	:	CUTANEOUS LEISHMANIASIS
MCL	:	MUCOCUTANEOUS LEISHMANIASIS
PCR	:	POLYMERASE CHAIN REACTION
APC	:	ANTIGEN PRESENTING CELL
MHC	:	MAJOR HISTOCOMPATIBILITY
DTH	:	DELAYED TYPE HYPERSENSITIVITY
iv	:	INTRAVENOUS
im	:	INTRAMUSCULAR
BCG	:	BACILLUS CALMETTE-GUERIN
NK-CELL	:	NATURAL KILLER CELLS
Ag	:	ANTIGEN
LACK	:	*LEISHMANIA* HOMOLOGUE OF RECEPTORS FOR ACTIVATED C KINASE
PMGL	:	PHOSPHATE MANNOGALACTAN LIGAND
FML	:	FUCOSE-MANNOSE GLYCOPROTEIN LIGAND
SUV	:	SMALL UNILAMELLAR VESICLE
MLV	:	MULTILAMELLAR VESICLE
Con-A	:	CONCANAVALIN A
RCA_{120}	:	RICINUS COMMUNIS AGGLUTININ
SBA	:	SOY BEAN AGGLUTININ
PNA	:	PEANUT AGGLUTININ
PHA-P	:	PHYTOHAEAGGLUTININ
PAS	:	PERIODIC ACID SCHIFF'S
PC	:	PHOSPHOCHOLINE

CHAPTER 1

LEISHMANIASIS: AN OVERVIEW

Parasitic infections are a major health problem world wide especially in under developed and developing countries. Several factors which are responsible for increasing parasitic diseases are population crowding, poor sanitation and health education, inadequate control of parasite vectors and reservoirs of infection, population migration and lastly development of resistance towards agents/drugs used for control of these diseases or vectors. In the recent past the World Health Organisation (WHO) has selected six important parasitic diseases in tropical and subtropical regions of the world for inclusion in its specific programme for research and training. It can be said that amongst the selected parasitic diseases, human leishmaniasis is second in importance only to malaria, as it possesses much greater problem both in treatment and control. Leishmaniasis is a general name given to the disease caused by infection with any member of the genus *Leishmania*. The current survey by WHO showed that about 12 million people are thought to be infected world wide with more than 400,000 new cases appearing each year (WHO, 1984; Anon, 1990) and approximately 350 million people are at risk (WHO, 1990; Ashford *et al.*, 1992)

HISTORY OF THE PARASITE:

Leishmaniasis includes a group of unicellular organism inhabiting macrophages of vertebrate hosts as an obligate parasite. They are transmitted from one host to another by a vector. sandfly (*Phlebotomus* spp). The natural hosts of *Leishmania* spp. are lizards, and other mammals, particularly canine, rodents, and primates including man.

The human leishmaniasis was first reported in 1885 by Cunningham in histological sections of oriental sore. The parasite was first named as *Piroplasma donovani*, which was subsequently changed to *Leishmania donovani* by Ross in 1903 (Zuckerman and Lainson, 1977) after its discoverers, Leishman and Donovan who reported the organism independently, Leishman in 1900 from London and Donovan in 1903 from Madras (Peters, 1988). Wright (1903) named the causative agent of oriental sore as *Helcosoma tropica* (Zuckerman and Lainson, 1977). Borovsky (1988) and Wright (1903), gave an accurate description of its morphology and was named *Leishmania tropica* by Luhe in 1906. Nicolle (1908) established the Trypanosomatid nature of *L. donovani* and *L. tropica*, and also gave the name *L. infantam* to the parasite which caused infantile Kala-azar. The Russian workers Yakimoff and Schockov in 1915 described *L. tropica* and *L. major*, the causative agent of zoonotic cutaneous leishmaniasis (Zuckerman and Lainson, 1977). *Leishmania braziliensis* was discovered in 1913 by Vianna and Muniz and Medina discovered *L. enriettii* in 1948 in Panama state (Zuckerman and Lainson, 1977). The western hemisphere parasites were classified into two major groups of *L. mexicana* and *L. braziliensis* complexed (Lainson and

Show, 1972). Visceral leishmaniasis was discovered in America in 1913 and the causative parasite was named *L. chagasi* by Margues Dacunha and Chagas in 1937 (Zuckerman and Lainson, 1977).

GEOGRAPHICAL DISTRIBUTION:

Leishmania donovani is a causative agent of Kala-azar or visceral leishmaniasis. It is widely distributed in Europe (Portugal, Spain, Italy, Malta, Greece, and Southern Russia), Africa, (Morocco, Algeria, Tunisia, Libya, Abyssinia, Sudan, Northern Kenya, Nigeria) Asia (India, China, Turkistan etc.) and in South America.

Leishmania tropica has been reported from Africa (mainly Mediterranean sea) Europe (Spain, Italy, France, and Greece), Asia (Syria, Palestine, Armenia, Southern Russia, Iraq, Iran, Arabia, Turkistan, India, Indo-China and China), and Australia (Northern (Queensland).

Leishmania Braziliensis (Vianna) has been reported from Brazil, Peru, Paraguay, Argentina, Uruguay, Bolvia, Venezuela, Ecuador, Colombia, Panama, Costa Rica, Mexico and South as well as Central America.

In India, the first available record of leishmaniasis was in 1862, and the disease was known as 'Jwar Vikar' in the local language of the district Jessore and took a total of 75,000 lives in three years (Sengupta, 1944). The disease is now highly endemic in the states of Bengal (Jayaraman, 1988), Orissa (Satyavati and Nando, 1987), Bihar (Thakur, 1984) and North west India (Naik *et al.*, 1979). Few cases have also been reported from Gujarat (Gajwani *et al.*, 1967),Kashmir (Jacob and Kalra,1951), Himachal Pradesh (Gupta and Bhatia, 1975), Chandigarh (Naik *et al.*, 1979), Madras (Sivaprakasam *et al.*, 1988), Mussourie (Chand *et al.*, 1988), Delhi and Uttar Pradesh (Kapur *et al.*, 1979).

TAXONOMY:

The genus *Leishmania* belong to the Phylum: protozoa, Subphylum: plasmodroma, class: Mastigophora, Subclass: Zoomastigina, Order: Protomonadina, Family: Trypanosomatidae, Genus: *Leishmania, Species: donovani, major, tropica, mexicana*, etc.

Simplified and traditional classification of leishmaniasis based on clinical manifestations are as follows.

1. **Visceral leishmaniasis or Kala-azar**: *L. donovani*, a chronic and often fatal disease which affects the macrophages of the liver, spleen, bone marrow and lymphnode.

2.**Cutaneous leishmaniasis or Oriental Sore**: *L. major, L.tropica, L. mexicana*, and *L. aethiopica*, which restrict their infection to dermal tissues only. This disease produces skin ulcers which leave an unsightly scare on healing. New World cutaneous leishmaniasis (NWCL) tends to be more severe and chronic than Old World cutaneous leishmaniasis (OWCL). Diffuse cutaneous

leishmaniasis (DCL) causes wide spread thickening of skin.

3. **Mucocutaneous leishmaniasis or Espundia**: *L. braziliensis*, which replicates primarily in mucous tissues and causes gross disfigurements. This affects mucosal region of nose, ear and mouth (Marr *et al.*, 1978). The details of clinical taxonomy is summarized in Table 1. The taxonomy of genus *Leishmania* was based primarily on clinical factors, differences in developmental vectors and virulence in experimental host. Since these criteria provide insufficient basis of classification. hence, techniques like monoclonal antibody (mAb) typing, recognising specific antigens and species specific probes (McMahon *et al.*, 1982), isoenzyme analysis (Kreutzer, 1980), DNA buoyant density (Chance *et al.*, 1974) and restriction endonucleases analysis of nuclear and mitochondrial DNA (Arnot *et al.*, 1981) have recently been used. The most common and so far most successful DNA identification method involves the use of kinetoplast DNA. It has about 10^7 base pairs of mitochondrial DNA, including approximately 10-20000 highly reiterated mini sequences of 500-2500 base pairs, used for identification of the species. (Barker, 1987)

MORPHOLOGY:

The *Leishmania* species are unicellular protozoa that exist in two distinct morphological forms. The flagellated promastigotes form, resides in the midgut and hindgut of alimentary tract of their insect vector (Sandflies). The parasite exists extracellularly as the flagellated, motile promastigote (Killick-kendrick and Robertson, 1977). Promastigotes, measure 15 to 20 um in length and 2 to 3 um in width. The fully developed ones are long slender spindle shaped body. They are monoflagellated parasite having nucleus, kinetoplast and eosinophilic vacuole etc.

The nonflagellated amastigote form is spherical or ovoid 2-4 um along the longitudinal axis. It contains a central rod shaped kinetoplast and axoneme which represents the root of the nonfunctional flagellum (Zuckerman and Lainson, 1977; Chang, 1983). The different morphological form of promastigotes and their developmental sites are described in (table 2) (L.L.Walter *et al.*, 1993)

LIFE CYCLE OF *LEISHMANIA* PARASITE :

The life cycle of *Leishmania* parasite consists of two different stages. In the first stage, the parasite lives as an extracellular flagellated promastigote form in the hindgut and midgut of alimentary tract of its insect vector, while in the second stage, as amastigote form residing in the cells of mononuclear phagocytic cells of the reticuloendothelial system of the mammalian host. The disease occurs when the insect vector (Phlebotomus argentipes) bites a healthy human host and injects the extracellular promastigotes into the blood stream. There, they encounter complement,

TABLE 1. TAXONOMY OF *LEISHMANIA* SPECIES KNOWN TO INFECT MAN

Species	*Disease*
Leishmania donovani	
L.d. donovani	Visceral (Kala-azar)
*L.d. infantum**	Infantile Visceral
*L.d. chagasi**	Cutaneous
Leishmania major	Cutaneous
Leishmania tropica	Cutaneous
Leishmania aethiopica	Diffuse cutaneous
Leishmania mexicana	
L.m. mexicana	Cutaneous
*L.m. amazonensis**	Cutaneous
L.m. pifanoi	Cutaneous
L.m. garnhami	Cutaneous
Leishmania braziliensis	
L.m. venezuelensis	Cutaneous
L.b. braziliensis	Mucocutaneous
L.b. guyanensis	Cutaneous
L.b. panamanensis	Cutaneous
Leishmania peruvana	Cutaneous

* Some consider these to be separate species.

Table 2. Proposed designation of *Leishmania* forms in the sand fly gut and developmental sites.

	Designation name	Location in gut
AM	Amastigote	Bloodmeal[a]
P1	First stumpy promastigote	Bloodmeal
P2	Second stumpy promastigote	Bloodmeal
P3	Third stumpy promastigote	Bloodmeal
Free-swimming phase[b]		
N1	Elongate nectomonad promastigote	Bloodmeal, midgut, hindgut
N2	Spatulate nectomonad promastigote	Bloodmeal, hindgut
N3	Short nectomonad promastigote	Midgut,[c] foregut?
N4	Nectomonad paramastigote	Midgut,[c] foregut?
N5	Metacyclic nectomonad promastigote	Midgut, foregut
Attached phase[d]		
H0	Pear-shaped haptomonad promastigote	Hindgut, foregut
H1	Elongate haptomonad promastigote	Hindgut, stomodeal valve?
H2	Spatulate haptomonad promastigote	Hindgut
H3	Short haptomonad promastigote	Stomodeal valve
H4	Haptomonad paramastigote	Hindgut, foregut

[a] The bloodmeal is surrounded by the peritrophic membrane.

[b] Parasites with a free flagellum in the lumen of the midgut, hindgut or foregut.

[c] In *Ph. papatasi*, this form of *Le. panamensis* develops inside the retained peritrophic sac.

[d] Parasites colonize the cuticular lining of the hindgut or foregut and are attached by a modified flagellum.

antibodies and phagocytic cells, all of which can kill promastigote (Hoover *et al.*, 1985; Pearson *et al.*, 1983). As many as 80% of the promastigotes are effectively killed by such mechanisms (Lewis and Peters, 1977). Shortly thereafter, the survivors can be found within the phagocytic cells, both neutrophils and mononuclear phagocytes. Different factors contribute to the attachment and uptake of the parasites into appropriate host cells. Long term survival is possible only in cells of macrophage family.

When infected sandfly takes blood meal, the infective promastigotes form present in its pharynx enter into the blood stream of the vertebrate host. Once this is inside the blood stream of the vertebrate host, promastigotes are phagocytosed by the mononuclear phagocytic cells of the host and transform into amastigotes which then begin replicating by binary fission within phagolysosomes. The host cells lyse, releasing free amastigotes. These amastigotes then infect the other cells (spleen, liver, bone marrow and lymph nodes) of reticuloendo-thelial system (Chatterjee *et al.*, 1957; Ghosh *et al.*, 1987).

The amastigotes are ingested by the sandfly during a blood meal from an infected vertebrate host. The parasite migrates to the midgut of the sandfly, where the amastigotes transform into promastigotes within 3-4 days. The flagellated parasite then gradually migrates forward to the pharynx by the fourth and fifth days. The transformation of amastigote to promastigote takes place in approximately one week (Figure -1). The promastigotes attach itself to the epithelial cell lining of the midgut of the sandfly and multiply. These are avirulent promastigotes. When promastigotes eventually cease dividing, they detach from the epithelial cells and migrate to the mouth part of the insect and these are termed as metacyclic promastigotes which are virulent form of the parasite (Sacks, 1988). The promastigotes need a basic environment (pH 7.0 to 7.5) with low temperature (24 ± 2°C) for growth. In contrast, the amastigotes perform their metabolic process, survival and multiplication at (pH 5.0 to 5.5) and high temperature of 37°C in 5% CO_2 (Chang *et al.*,1985).

HOST PARASITE INTERACTIONS:

There are several factors which regulate the attachment of promastigotes to the phagocytic cells. Initially, infection is dependent on the ability of promastigotes and later on amastigotes. Some problems are involved during interaction, like, homogeneity of promastigotes population (Sacks and Perkins, 1984; 1985), differentiation and activation of host cell receptors (Tait and Sacks, 1988), parasite differentiation from non-infective to infective stage in the alimentary canal of the midgut of sandfly (Sacks, 1990). In spite of these problems, parasites do succeed in developing infection in host cells. The *Leishmania* promastigotes bind and are internalize by all phagocytes, but they survive only in macrophages and less mature monocytes. Interaction of *Leishmania* promastigotes with macrophages is a receptor mediated event. The promastigotes bind

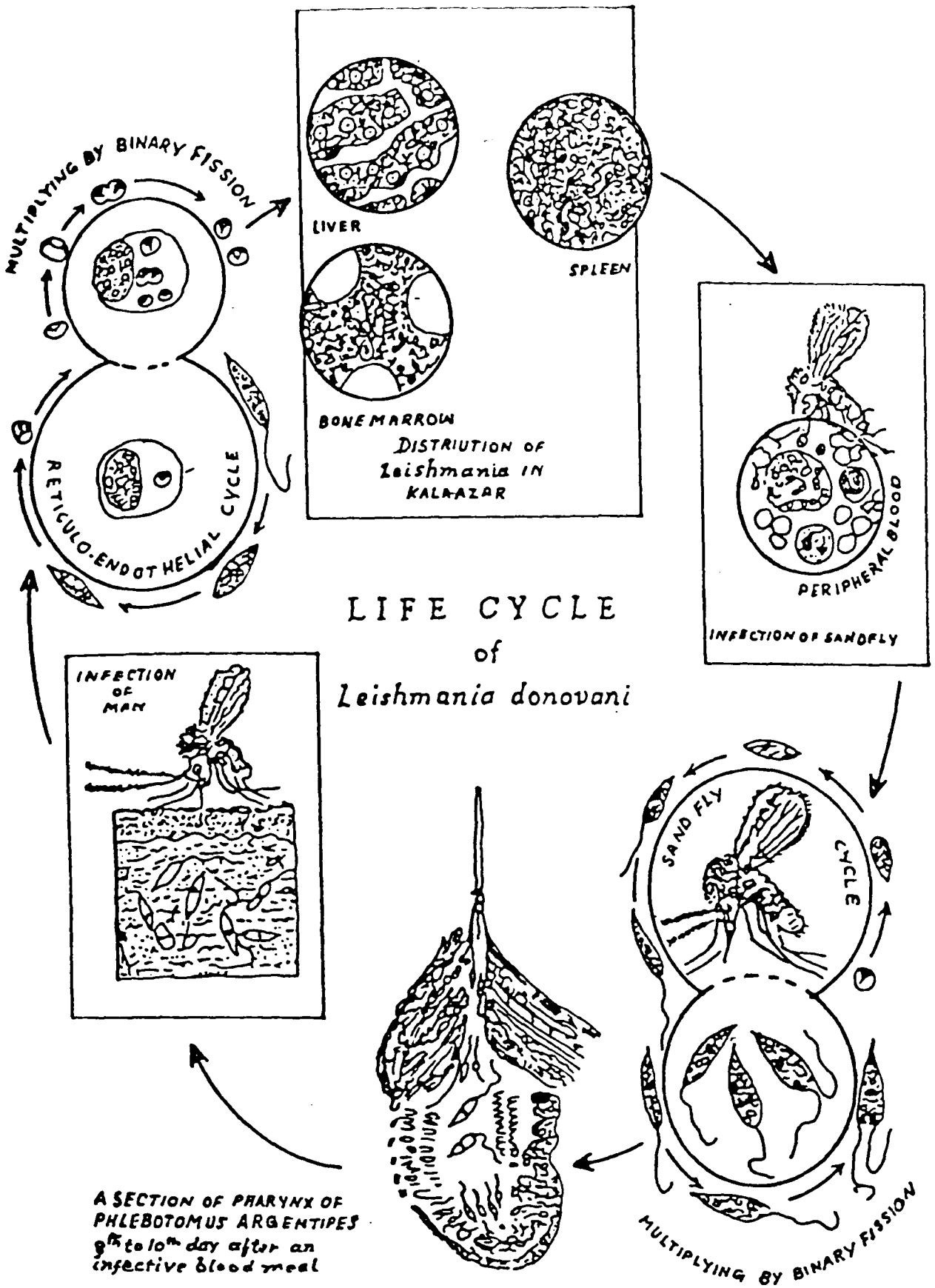


Figure 1: Life cycle of *Leishmania donovani* showing reticuloendothelial system, distribution of *Leishmania* in Kala-azar and sandfly cycle (Chatterjee, 1957).

directly to macrophage receptors or through the intervention of the host factors, i.e., complement receptors (CRI/CR 3), mannose-fucose receptor (MFR) and fibronectin receptor (FnR) (Mosser and Edelson, 1984; 1985; 1987; Russell *et al.*, 1989). Promastigotes may utilize several different macrophage receptors depending on the parasite species and developmental stage and on the presence or absence of serum. In the presence of serum, however, the promastigotes surface is opsonized with complement component such as C3b and iC3b, and promastigotes uptake occurs via the complement receptors CR1 or CR3 (da Silva *et al.*, 1989; Mosser *et al.*, 1992). The host parasite attachment in the presence of serum factors of the mammalian host, may also involve, natural antibodies, oxidative metabolites, and digestive fluids or secretory products of salivary glands of the sandfly vectors.

It has been demonstrated that surface molecules of parasite like 63kDa glycoprotein (gp63) and lipophosphoglycan (LPG), bind directly to a number of macrophage receptors in the absence of serum (Handman and Goding 1985; Chang and Chang, 1986; Russell and Wilhelm, 1986) (Fig. 2). Purified gp63 has been shown to bind to the macrophage receptors as its binding to macrophage was blocked with anti-CR3 monoclonal antibodies (Russell and Wright, 1988). Thus, the binding of gp63 is mediated by CR3 receptor of the macrophages (Russell, 1990). The binding of LPG to macrophages was first demonstrated by Handman and Goding, 1985. Differential inhibition of individual members of the macrophage receptor indicated that CR3 and $P^{150/95}$ were primarily responsible for LPG binding (Talamas-Rohana *et al.*, 1990). LPG undergoes extensive elongation during differentiation of procyclic promastigotes to the metacyclic promastigotes. This alteration leads to increased complement resistance of *Leishmania* parasites and a thick coat is formed on the parasite surface (7nm in log phase-17nm in stationary phage) (Sacks and da silva, 1987; Pimenta *et al.*, 1989; da Silva *et al.*, 1989). The MFR of macrophage may also binds to the LPG and help in parasite phagocytosis. It has been observed that lectins like receptors are present in the midgut of sandfly that act as hemagglutinins and are inhibited by only three sugars (galactosamine, glucosamine, mannosamine). The phosphorylated tetrasaccharides, effectively inhibit (>70%) the binding of procyclic promastigotes to *P. papatasi* (Pimenta *et al.*, 1992). LPG has been shown to be a ligand to the sandfly midgut epithelium as only *L. major* strain having complete procyclic LPG was found to be attached to the midgut of their natural vector, *P. Papatasi*, while *Leishmania* of other species like, *L. donovani*, *L. tropica* and *L. amazonensis* failed to attach to the epithelium cell lining of midgut of *P. papatasi*. Contrary of this, *P. argentipes* a natural vector of *L. donovani* supported the growth of *L. donovani*, *L. major*, *L. tropica* and *L. amazonensis* promastigotes in the epithelium of sandfly midgut. Furthermore, *L. donovani* mutant deficient in LPG failed to attach to the midgut of this sandfly. The multiple β linked galactose residue of *L. major* LPG was found to be responsible for the attachment to the epithelial cell line of midgut of *P.*

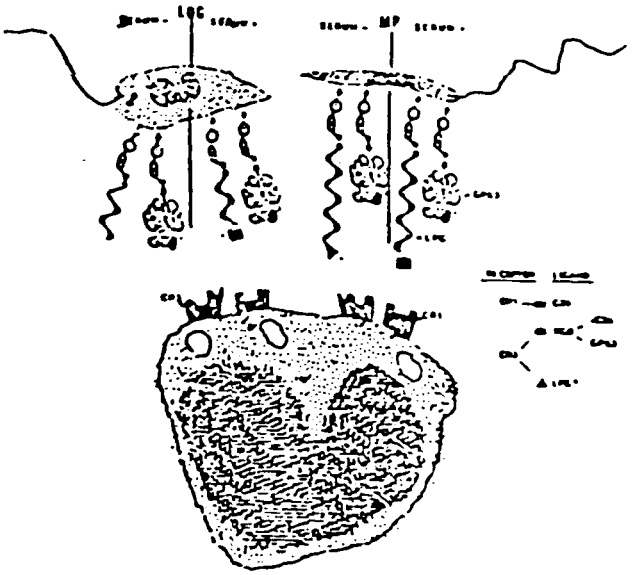


Figure 2: Receptor ligand interactions between *Leishmania* major promastigotes and human macrophages. The two major cell surface glycoconjugates, LPG and gp63 are depicted as controlling the attachment of different developmental stages of promastigotes on which the expression of the LPG is developmentally modified. Multiple interactions might be required for subsequent events associated with receptor activation and internalization (Tait and Sacks, 1988).

papatasi and was found to be species specific. Contrast to this the *P. argentipes* midgut possesses a receptor for relatively conserved oligosaccharides or 'lectin like' molecules of procyclic LPG of different species of *Leishmania* parasites. These observation suggesting that LPG is a ligand to the sandfly midgut epithelium and plays an important role in host parasite interaction (Pimenta *et al.*, 1994; Volf *et al.*, 1994; Sacks and Turco, 1995). Acid phosphatase is also a cell surface molecule and reported to be secreted in the culture medium, (Dwyer and Gottlieb, 1985; Glew *et al.*, 1988). Two form of acid phosphatase are present, a membrane bound, tartrate resistant and other a secretory acid phosphatase. They are antigenically distinct and exist as multiple isoenzymes present in both stages of most *Leishmania spp.* It has been shown that acid phosphatases dephosphorylate certain phospholipids and phosphoproteins which may provide the parasite a source of energy as inorganic phosphate for growth. *Leishmania* spp. possess a superoxide dismutase which converts $O_2^{\cdot}$ to H_2O_2, which can diffuse into the extracellular spaces and *kill Leishmania* parasites. Promastigotes are generally more sensitive than amastigotes to H_2O_2 and $O_2^{\cdot}$ (Murray, 1981; Pearson *et al.*, 1983). Thus, this ectoenzyme is thought to protect *Leishmania* spp. by interfering with regulatory mechanism of the macrophages that produces microbicidal free radicals (Glew *et al.*, 1988).

The mechanism of host resistance and susceptibility are dependent on the T_H1/ T_H2 type response and macrophage-cytokine interactions. The resistance correlates with the T_H1 cell response, while susceptibility correlates with a T_H2 cell response (Maingon *et al.*, 1995; Kemp *et al.*, 1996). Production of IFN-γ by T_H1 cells has been found to be essential for macrophage activation, microbial clearance, healing and protective immunity in *Leishmania* infection (Belosevic *et al.*, 1989). Conversely, nonhealing response in susceptible mice have been related to the expression of the T_H2 subset and the production of cytokines such as IL-4 and IL-10 (Heinzel *et al.*, 1989; Locksley *et al.*, 1991). IL-12 and IFN-γ are known to be associated with the healing process and also play an important role in immunity against human visceral leihmaniasis (Ghalib *et al.*, 1995). The production of both IL-12 and IFN-γ is inhibited by IL-10. It has also been demonstrated that the disruption of IL-4 gene can confer protective immunity by T_H1 type immune response, as determined by positive DTH and IFN-γ production (Satoskar *et al.*, 1995). In addition, production of nitric oxide has been found to be most likely an effector mechanism for parasite killing. (Bhakuni *et al.*, 1996; Stefani *et al.*, 1994; Bogdan *et al.*, 1993).

PATHOGENESIS AND SYMPTOMS:

Leishmania is an obligate intracellular parasite that cause a broad spectrum of diseases in man and other animals, ranging from self-healing skin ulcers to a fatal visceral infection.

The *Leishmania* parasite after entering the host, invades the cells of the reticuloendothelial system where it resides and multiplies. Thus, the parasites are distributed all over the body and are particularly found in tissues rich in reticuloendothelial cells. They multiply in the reticuloendothelial cells, specially the macrophages in spleen, liver, lymph nodes and bone marrow, which become heavily parasitized. The initial infection and the appearance of the clinical manifestations are generally observed from three weeks to six months (Thakur, 1984), but the incubation period may extend upto 2-9 years (Faust and Russell, 1964). Early stage of visceral leishmaniasis is characterized by malaise, headache, and fever occurring at irregular intervals later becoming regular and accompanied by chills and sweating. Chronic visceral leishmaniasis produces marked enlargement of liver and spleen (hepatospleenomegaly). The spleen is more enlarged as compared to liver. The other symptoms are high fever, pyrexia, lymphadenopathy, anaemia, the skin over the body becomes dry, rough and harsh and is often pigmented (darkened) (Sen Gupta *et al.*, 1956; Khalid *et al.*, 1990). The hair tends to be brittle and fall off. There is a reversal of normal albumin-globulin ratio. In the active stage of disease, the peripheral blood (T/B cell) ratio is also reversed (Rezai *et al.*, 1977). Kala-azar patients are also found to have a defective cellular immunity, as detected through skin test (Manson Bahr, 1961). Lesions in the bronchi and lungs, intestinal ulcers and hemorrhages in the internal organs are also reported to occur simultaneously. In the later stage of disease the skin usually becomes black in colour and there is continuous high fever, justifying the name of the disease 'Kala-azar' (Black sickness). If patients are untreated, 75-95% die within a period of two years. Death in Kala-azar is always due to secondary infections, such as amoebic or bacillary dysentery, pneumonia, pulmonary tuberculosis, cancerum oris and other septic infection (Nag and Ghosh, 1955). Due to the suppressed immune system tuberculosis and AIDS are opportunistic infection in Kala-azar patients (Fuzibet *et al.*, 1988, TDR News No. 36, 1991).

In about 20% of Indian Kala-azar patients, there are non-ulcerative nodular cutaneous lesions known as post Kala-azar dermal leishmaniasis (PKDL), generally occurring one or two years after the completion of sodium stibogluconate treatment. PKDL lesions have also been found in some spontaneously cured Kala-azar cases (Rees and Kagar, 1987; Chessborough, 1988).

DIAGNOSIS OF KALA-AZAR:

Clinical diagnosis of the kala azar on the basis of signs and symptoms (prolonged fever, hepatospleenomegaly, abdominal distension, pallor, anorexia, anaemia, leucopaenia, thrombocytopaenia and hyperglobulinemia, etc.) is very difficult because these symptoms may also be present due to other diseases. Therefore, parasitological confirmation before treatment is essential. Parasites may be demonstrated in the aspirates of spleen, bone marrow and lymph nodes. The demonstration of the *Leishmania* parasites by (direct evidence method) blood smears or blood

culture and biopsy of the parasitized tissues is possible and biopsy of bone marrow and spleen are still used today. Although spleen aspiration is a high risk technique and there is no consensus on its use, however, because of its superior sensitivity it is the preferred method. The bone marrow puncture, which is widely practiced, is less hazardous and easy to perform than spleen aspirates, is more painful and labour extensive. Bone marrow aspirated samples are *cultured in vitro* for propagation of the parasite and also smeared on microslides for microscopic examination. Blood smears are generally negative because of low levels of circulating parasites. Blood culture method is time-consuming, and less sensitive. Therefore, it is neither preferred nor used now a days.

A number of serological, immunological and DNA based techniques are now being applied for the diagnosis of Kala-azar. The most widely applied immunological test in leishmaniasis has been the leishmanin or Montenegro's skin test. Which is usually positive in CL and MCL. It is negative in acute VL and most cases of PKDL but becomes positive after recovery. It is also negative in DCL. The leishmanin reaction is not species specific (Show and Lainson, 1967; 1974; 1975; Southgate and Manson Bahr, 1967). The skin test could be used for evolution for the effective immunization with killed *Leishmania* promastigotes (Mayrink, *et al.*, 1978) or leishmaniazation applied in the control of cutaneous leishmaniasis (Nadim *et al.*, 1983). Some serological tests based on specific antibody or antigens present in the serum, such as ELISA, have been developed. This is widely used and is most suitable and sensitive technique for detection of Kala-azar. ELISA has the advantage of being useful for both antigen and antibody detection. The Dot-blot ELISA test is similar to ELISA but the antigens are used on paper or plastic support to detect antibody which is then visualized using antiglobulins labelled with enzymes (Voller, 1993).

Diagnosis method based on parasite DNA are very useful but still face a number of limitations as it requires specific DNA probe and sophisticated instrument facilities. Recently a more sophisticated polymerase chain reaction (PCR) technique, is being used for detection of Kala-azar (Salkl *et al.*, 1988; Audya *et al.*, 1992). In PCR, a specific target DNA fragment is enzymatically amplified from total DNA or RNA of given organism using a thermostable DNA polymerase and oligonucleotide primers flanking the target site. The specificity of the primers normally ensures that other DNA sequences are not amplified during amplification. The main advantage of PCR is greater sensitivity for diagnosis of Kala-azar, very low level of parasitemia can be detected by this. At present PCR has no field application since the technique requires high skill and also sophisticated equipments.

IMMUNITY TO THE VISCERAL LEISHMANIASIS:

In human infections, the specific immune response to the pathogen may be of vital importance for host defense. An inappropriate response may not only result in lack of

protection, it may even contribute to the pathology of disease. After infection with *Leishmaia* parasites, humans often develop immunity to reinfection. The protective immunity against both visceral (Skov and Twohy, 1974 a;b) and cutaneous leishmaniasis (Preston and Dumonde 1976) is a T-cell mediated process. Generally the killing of amastigotes inside the macrophages requires the release of T-cell factors that lead to activation of the macrophages and produce more of the lethal substances which eventually kill the parasites. If T-cell activation and lymphokine production does not occur, then the macrophages will not be able to kill the parasites (Leiw, 1990). T-lymphocytes can recognise antigens associated with class I and class II MHC molecules i.e. $CD8^+$ and $CD4^+$ T-cells respectively, on the surface of accessory or antigen presenting cells (APCs). Leishmanial antigen has been demonstrated on the surface of infected macrophages (Farah *et al.*, 1975; Berman and Dwyer, 1981; Handman and Hocking, 1982), but it has not been associated with the concomitant expression of class II molecules.

The production of co-stimulatory factors, including IL-1, IL-2, IFN γ by the accessory cells promote antigen specific lymphocyte activation (Unanue, 1984; Kaye, 1987; Unanue and Allen, 1987; Weaver and Unanue, 1990; Kaye *et al.*, 1991). An adherent cell populations (macrophages) have been found to be associated with immunosuppression in leishmaniasis (Scott and Farrell, 1981; Reiner and Malemude 1984; 1985; Murray *et al.*, 1986). Impairment of the immune response has been found to be associated with inability of macrophages to produce IL-2 upon specific or mitogenic stimulation (Reiner and Finke, 1983; Cillari *et al.*, 1986; Murray *et al.*, 1987).

An antigen specific unresponsiveness is common during active visceral leishmaniasis. It is associated with reduced IFN-γ and IL-2 production (Carvalho *et al.*, 1984a; Kaye *et al.*, 1991). However, the absence of these co-stimulatory factors in chronic infected mice with *L. donovani* resulted in the failure of providing the necessary signals to activate IFN-γ producing T-cells. So far, IL-1 has been identified as co-stimulatory factor for activation of T_H2 cells, but not T_H1 cells which induced T_H2 cell proliferation and disease exacerbation. (Kurt Jones *et al.*, 1987). Freund's complete adjuvant is a potent stimulator of T_H1 cells (Gun and maurer, 1989), which can promote a protective response against PT3, a T-cells epitope derived from the primary structure of *L. major* gp63 (Jardim *et al.*, 1990). PT3 is found to activate IL-2 and IFN-γ producing T-cells in presence of adjuvant, while PT3 alone is able to induce T_H2 cell and these cells produced more IL-1. Therefore, disease exacerbation and protection depend on the expression of T_H2 and T_H1 cells, respectively (Fig. 3).

Humoral immunity:

Immune responses, including antibody, complement and phagocytes, participates in the destruction of promastigotes that enter the body. The local production of antibody at the site

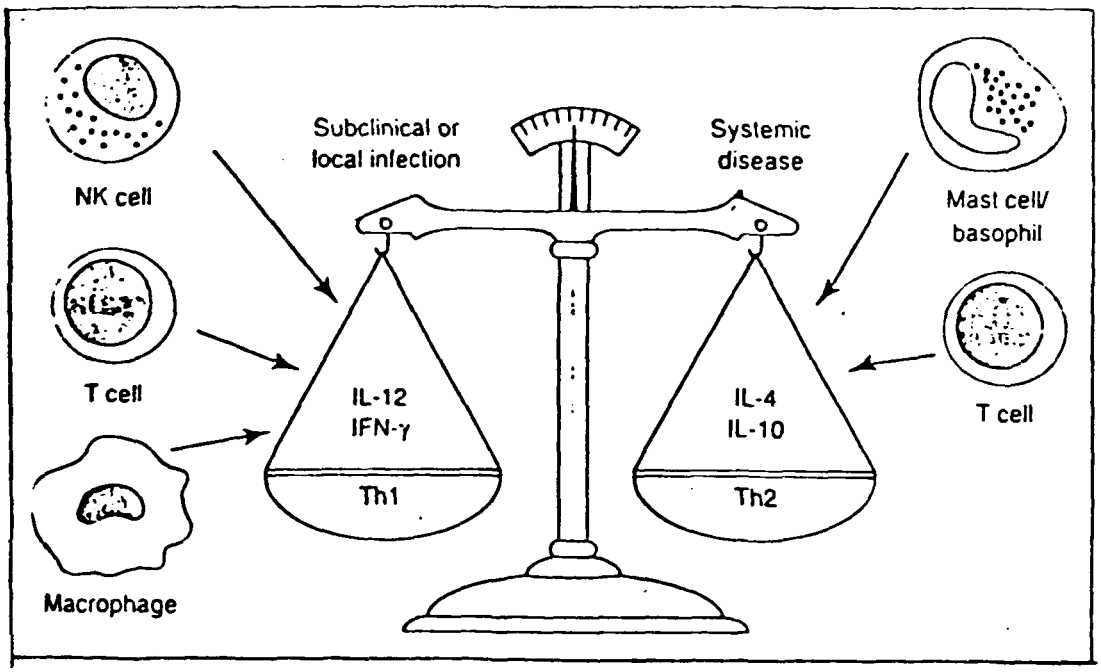


Figure 3: The development of antigen-specific responses by T-cell subset in humans infected with *Leishmania* depends upon balance of TH_1 and TH_2 cells which are responsible for disease resistance and disease exacerbation. In addition, cytokines produced locally by non specific cells may provide an environment that favours development of either TH_1 or TH_2 responses (Kemp *et al.*, 1996).

of infection have been shown by presence of plasma cells in leishmanial lesions (Stabur *et al.*, 1970). A few studies had revealed the production of IgG1, IgG3, IgE and IgA during the course of infection of leishmaniasis (EL-Amin *et al.*, 1986; Lunch *et al.*, 1986). Clinically, non-healing visceral leishmaniasis have been associated with high immunoglobulin levels and negative DTH, while healing or cured individuals display strong DTH and low levels of antibody (Turk and Bryceson, 1971). Antibodies may also influence the course of infection by directly affecting parasite-macrophage interactions. The potential protective functions of antibodies have also been demonstrated *in vivo* (Anderson *et al.*, 1983). A monoclonal antibody against a surface glycoprotein (M-2) of *L. mexicana* has been shown to be protective, if inoculated into the footpads of BALB/c mice along with parasites. Vaccination with M-2 (glycoprotein) plus adjuvant also induce protection, which is associated with increased antibody levels (Champsi and McMahon-Pratt, 1988; Reiner and Locksley, 1995; Liew and O'Donnell, 1993).

Cellular immune response:

Not only humoral immunity plays a role in *Leishmania* infection but cell mediated immunity also plays a major role in protection. The more protective immune response for the resolution of *Leishmania* infection is the cellular reactions (Pearson *et al.*, 1983; Liew, 1986; 1989). Control of the infection is influenced by the induction of immune responses in various T-cell subpopulations. There may be preferential induction of protective T-cell reactions. The protective T-cell secretes γ-interferon (IFN-γ), which is a potent activator of macrophage microbicidal activity (Liew, 1989). Although interferon acts by stimulating macrophages to activate antimicrobial activity, but other lymphokines are, also necessary for the efficacy of interferon (Davis *et al.*, 1988). During early infection, all types of lymphokines are produced, but as infection progresses, either the protective or the disease-promoting responses take precedence (Scott *et al.*, 1988). Recently it has been demonstrated that a balance between T_H1 and T_H2 cells is responsible for disease exacerbation or resisitance (Kemp *et al.*, 1996) (Figure 3).

(I) $CD8^+$ T cells mediated immunity:

The T-cells conferring protection or counter protection primarily belong to the $CD4^+$ T-cell subset (Mitchell *et al.*, 1981; Liew *et al.*, 1982; Louis *et al.*, 1982; Gorczynski, 1985). $CD8^+$ T-cell plays a protective role in leishmaniasis. Administration of anti $CD4^+$ monoclonal antibodies increases resistance to *L. major* (Titus *et al.*, 1985; Hill *et al.*, 1989) while administration of anti $CD8^+$ monoclonal antibodies exacerbated infection *in vivo* condition in mice (Farrell *et al.*, 1989). A protective role of $CD8^+$ T-cell subset during *L. donovani* infection is found which is associated with the inhibition of parasite growth in hepatic nodules (Mc Elrath *et al.*, 1988). However, $CD8^+$ T-cells can produce IFN-γ upon specific stimulation (Kaufmann 1988; Salgame *et al*, 1992), and

activate macrophages to kill *Leishmania* parasites (Smith *et al.*, 1991).

(II) $CD4^+$ T-cells mediated immunity:

The resistance and susceptibility of the parasites are mediated by at least two different $CD4^+$ T-cell subsets (Scott,1989; Liew,1989). These are the T_H1 and T_H2 $CD4^+$ T-cell subsets which are morphologically identical, but functionally distinguishable, on the basis of cytokines production (Mosmann *et al.*, 1986; Cherwinski *et al.*, 1987). T_H1 cells produce IFN-γ and IL-2, and mediate DTH and IgG2a antibody production, while T_H2 cells produces IL-4 and IL-5, and promote IgE and IgG1 production but do not mediate DTH. Several other cytokines including IL-3 and Granulocyte-macrophage colony- stimulating factor (GMCSF) are produced in varying amounts by both cell types. Based on the various studies on the *Leishmania* resistant and susceptible mice it has been suggested that the T_H1 cells have a protective role and T_H2 cells are counter protective. The protective T-cell lines and clones secreted IFN-γ and IL-2, while the counter protective T-cell lines and clones secreted IL-4 and IL-5. Healing and resistance are invariably associated with IFN-γ and IL-2 secretion, and susceptibility and non-healing with IL-4 and IL-5 production. The IL-3, which is produced by both T_H1 and T_H2 cells, has been shown to be counter protective (Scott *et al.*, 1989; Muller and Louis, 1989; Alexander and Russell, 1992; Rollinghoff, 1990).

III) Lymphokines/cytokines mediated immunity:

Killing of *Leishmania* parasites by IFN-γ and TNF-α in activated macrophages have been demonstrated. TNF-α and IFN-γ act synergistically to activate macrophages leading to the killing of *Leishmania* parasites (Liew *et al*, 1990; Paul, 1993). The leishmanial protection mediated by IFN-γ has been shown to be inhibited by IL-3 (Liew *et al.*, 1989). Pretreatment of macrophage with IL-4 inhibits macrophage activation and parasite killing mediated by IFN-γ , while treatment of infected macrophages suggested that IL-4 enhances IFN-γ mediated leishmanicidal activity (Phillipes *et al*, 1990). Thus, interplay between IL-4 and IFN-γ in inducing macrophage for leishmanicidal activity is very complex. IL-4 act synergistically with IFN-γ to promote macrophage activation resulting in release of TNF-α , which activates the leishmanicidal activity of macrophages (Solbach *et al*, 1991).

Recent studies have suggested that IFN-γ has a capacity to enhance host defense in euthymic animals (Murray, 1988; 1994). Antileishmanial activity *in vitro* or *in vivo* in euthymic mice can be induced by regulated T-cells or can be modulated by IFN-γ (Murray *et al.*, 1993;1995; Murray, 1995; Tumang *et al.*, 1994; Liew *et al.*, 1990). IFN-γ can directly activate macrophages *in vivo* regardless of the host's complement of T-cells and indicates that $CD4^+$ or $CD8^+$ T-cells can act with IFN-γ to activate intracellular microbial activity. Based on these findings it was suggested that for most T-cell-deficient patients, including AIDS patients, with preserved $CD8^+$ cells, an adjunctive

therapy with IFN-γ should be explored for opportunistic intracellular infection.

In addition to IFN-γ, *in vivo* responsiveness to exogenous administration of other cytokines like, TNF-α , IL-1, IL-2, IL-7, IL-12 and GM-CSF also appears to require participation of host T-cells for optimal induction of antitumor or non-viral antimicrobial activity (Murray *et al.*, 1993; 1995; Murray and Hariprasad, 1995; Brunda *et al.*, 1993; Hock *et al.*, 1991; North *et al.*, 1988; Aukerman *et al.*, 1990).

IL-10, a T_H2 type associated cytokine, inhibits antigen-specific cellular responses in active visceral leishmaniasis. It has been shown to induce many inhibitory effects on IFN-γ production and function on the macrophage capacity of antigen presentation and cytotoxicity (Moore *et al.*, 1990; Bogdan *et al.*, 1991; De Waal Malefyt *et al.*, 1991). IL-10 was shown to inhibit human lymphocyte IFN-γ production, by suppressing NK-cell stimulating factor.

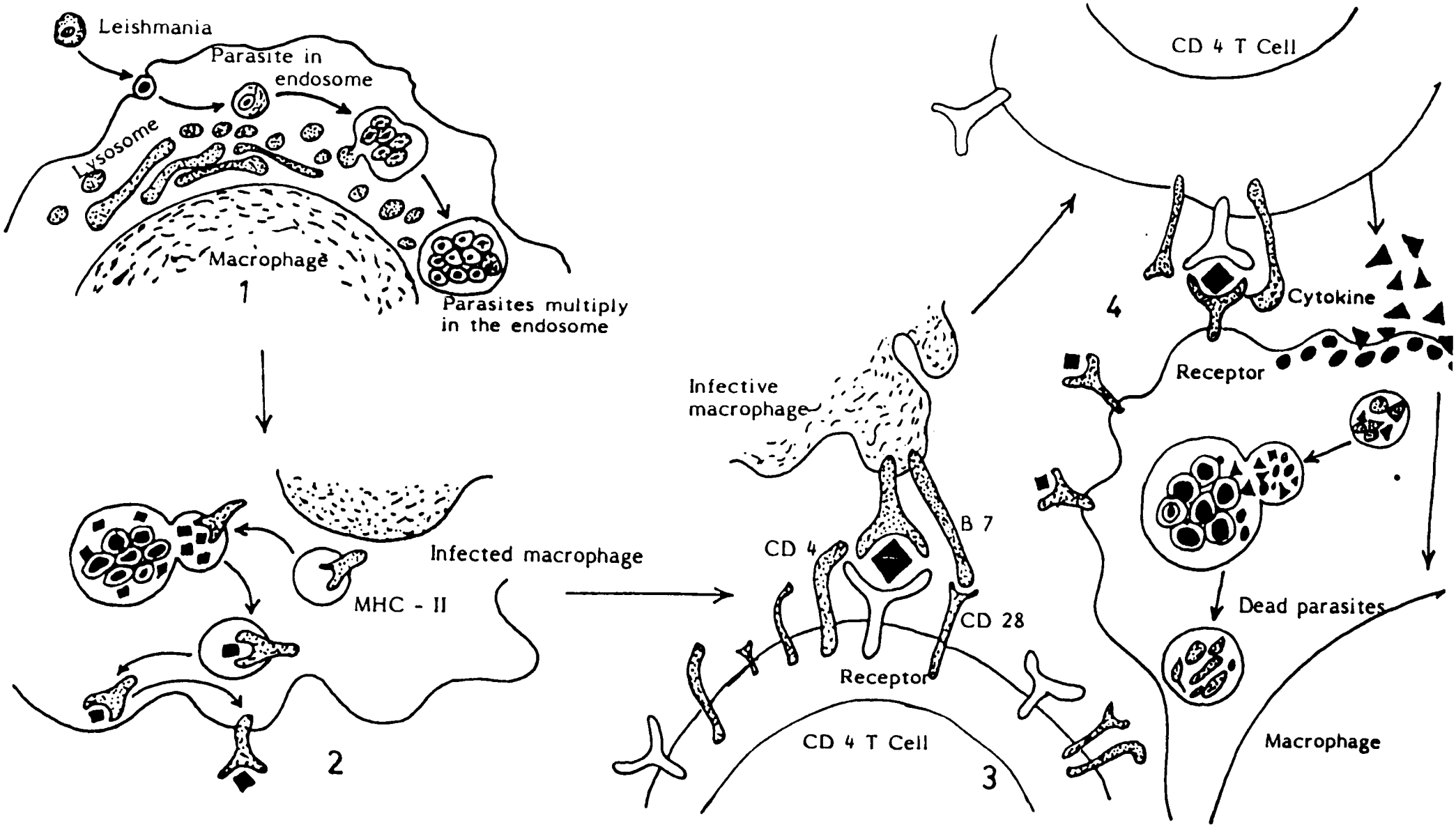
IL-12 is a pluripotent cytokine that interacts with NK and T-cells to play a central role in the initiation and maintenance of T_H1 responses and IFN-γ production and is associated with cure of *L. major* infections (Heinzel *et al.*, 1993; Scott, 1993; Sypek *et al.*, 1993; Chan *et al.*, 1991). The production of IFN-γ by T_H1 cells appears to be essential for macrophage activation, microbial clearance, healing and protective immunity in leishmanial infection (Belosevic *et al.*, 1989). The production of IL-12_p40 by PBMC from treated patients has been reported to be markedly reduced by addition of anti-IFN-γ (Kubin *et al.*,1994). The production of these two cytokines after successful treatment was found to be interdependent. This provides evidence that IL-12 and IFN-γ are associated with healing process and could play an important role in immunity against human visceral leishmaniasis (Finkelman *et al.*, 1994; Zhang *et al.*, 1994; Ghalib *et al.*, 1995; Murray *et al.*, 1995; Kemp *et al.*, 1996, Tremblay et al. 1996, Milon et al, 1995, Solbach and Laskay, 1995) Figure 4.

(iv) Reactive Oxygen Species:

The toxic metabolites of oxygen, superoxide (O^-_2), singlet oxygen ($^1O_2$) the hydroxyl radical(OH) and most especially hydrogen peroxide (H_2O_2) have been thought to be responsible for macrophage leishmanicidal activity (Murray, 1981; Pearson *et al.*, 1982; da Silva *et al.*, 1989). Evidence for this view point arose because of studies that demonstrated that amastigotes of *L. donovani* and metacyclic promastigotes of *L. major* survived better than, and triggered the macrophage respiratory burst only weakly compared with, log phase promastigotes. This ability was also attributable to amastigotes having higher levels of glutathione peroxidase, superoxide and catalase than promastigotes (Murray, 1982; Pearson *et al.*, 1983). Intracellular amastigotes are also capable of down-regulating the macrophage oxygen-dependent microbicidal potential (Buchmuller-Rouiller and Mauel, 1987). Nevertheless, there has been increasing evidence from several studies

Figure 4: How the immune system fights *Leishmania* parasites:

1. *Leishmania* parasites proliferate inside the organelles of cells, where they a beyond the reach of antibodies. Macrophages ingest *Leishmania* organisms a hold them in vesicles called endosomes. Many organisms trapped in endosom are later destroyed by enzymes from lysosomes. *Leishmania* parasites, howev survive and multiply in the endosomes. (Paul, 1993).
2. Class II MHC molecules produced by the infected macrophage bind to pepti from the parasite. The MHC molecule carries the peptide to cell surface.
3. $CD4^+$ T cells that have complementary receptor molecules are activated by t peptides class II complex and by the B_7 molecule on the surface of t macrophage.
4. The activated T cells secrete certain cytokines that bind to receptors on t macrophage. In response the macrophage produces TNF, NO and other fact that kill the parasites.


Leishmania
Parasite in endosome
Lysosome
Macrophage
1
Parasites multiply in the endosome
Infected macrophage
MHC - II
2
Infective macrophage
CD 4
B 7
CD 28
Receptor
CD 4 T Cell
3
CD 4 T Cell
4
Cytokine
Receptor
Dead parasites
Macrophage

that oxygen-independent mechanisms are capable of killing *Leishmania*; not only are *L. donovani*, *L. mexicana*, and *L. major* resistant to oxygen radicals (Pearson *et al.*, 1982; Mallinson and Coombs, 1989b), but they can be killed by macrophages deficient in the production of oxygen metabolites (Murray and Cartelli, 1983; Scott *et al*, 1985).

Recently a new mechanism of macrophage anti-leishmanial killing has been characterized as a novel metabolic pathway synthesizing nitric oxides [nitric oxide (NO), nitrite oxide (NO^-_2) and nitrate oxide (NO^-_3)] from L-arginine with L-citrulline as a co-product (Green *et al.*, 1990; Liew *et al.*, 1990a,b).

Nitric oxide is derived from the guanidinonitrogen of L-arginine and molecular oxygen in a reaction catalyzed by the enzyme nitric oxide synthase (NOS) (Palmer *et al.*, 1987; Marletta *et al.*, 1988; Hibbs *et al.*, 1988). Nitric oxide has been shown to play an important role in the killing of the protozoan parasites of *Leishmania* species (Liew and Cox,1991; Mauel *et al.*, 1991; Roach *et al.*, 1991; Bogdan *et al.*, 1993; Paul *et al.*, 1993; Stefani *et al.*, 1994). Macrophage NO mediated killing of *L. major* has been shown to be induced by TNF-α acting synergistically with IFN-γ (Liew *et al.*, 1990a). This biochemical pathways, as well as anti *Leishmania* activity, is inhibited in the presence of D-arginine and N-monomethy L-arginine (Liew *et al.*, 1990b; James and Hibbs 1990).

TREATMENT OF LEISHMANIASIS:

The World Health Organization (WHO) estimates 12 millon cases of leishmaniasis worldwide, with annual figures of one million for visceral leishmaniasis (VL) and three million for cutaneous leishmaniasis (CL) (Anon, 1990). Cutaneous leishmaniasis is a self healing and relatively benign disease but visceral leishmaniasis or kala-azar, is a wide spread, severe and often fatal disease in the absence of specific chemotherapy. Several chemotherapeutic agents against human visceral leishmaniasis are available.

CHEMOTHERAPY OF LEISHMANIASIS:

Antimonials Drugs:

The antimonial drugs are most suitable for chemotherapy of leishmaniasis. These are known to be tissue specific in their action. They accumulate most rapidly in liver where their efficacy is higher as compared to that in spleen (Collins *et al.*, 1992). The antimonial drugs are divided into two main groups: trivalent and pentavalent antimonials. Trivalent antimonial (urea stabamine) is now rarely used for the treatment of leishmaniasis. Pentavalent antimonials are as follows:

Pentavalent antimonials:

The important pentavalent drugs are :

(I) Sodium stibogluconate (Pentostam)

(II) Meglumine antimonate (Glucantime)

(III) Neostibosan (Ethyl stibamine)

(IV) Urea stibamine (Corbostibamine, stiborea) and derivatives of stibanilic acid such as stibacetin.

Among these, sodium stibogluconate and meglumine antimonate are drugs of choice till date. Sodium stibogluconate (Pentostam Sb^v) developed by Wellcome foundation. UK, in 1925 was first used in China with great success. Pentostam is more potent and well tolerated antimonial drug (Berman, 1988: Thakur *et al.*, 1988), which is used for visceral leishmaniasis (Black *et al.*, 1977; Chulay *et al.*, 1988; Thakur *et al.*, 1988; 1990). The recommended dose of Sb^v is 10-20 mg/kg/day (im or iv) for 20-40 days depending on the geographical distribution and, body surface area of patients and also to reduce unresponsiveness to the drug (Thakur *et al.*, 1988; 1989; Olliaro and Bryceson, 1993;Chulay *et al.*, 1983; Chance, 1995).

The drawback of Pentavalent antimonial is their rapid excretion by the kidneys, which is about 60-80% within six hrs of administration. This leads to acute toxicity, perhaps due to the production of trivalent metabolites, which increases in proportion with dose and duration (Bryceson, 1987). Besides incomplete cure, the usual side effects are vomiting, diarrhoea, abdominal pain, anorexia, malaise, itching, fever, dizziness, headache, cardiotoxicity and nephrotoxicity with pentostam have also been reported (Chulay *et al.*, 1985; Ree *et al.*, 1985; Karen *et al.*, 1987).

In cutaneous leishmaniasis (CL), the parasites are relatively insensitive to antimonials, as the concentration of the drug tends to be more in liver and spleen (Bryceson, 1987),than reaching in desired concentration to skin(Thakur and Kumar,1990; Herwaldt and Berman, 1992). Mucocutaneous leishmaniasis (MCL) is relatively poor responder to antimonials, possibly due to the site of infection, poor immune response and partial drug resistance to antimonials (Grogl,1989).

Meglumine antimoniate (Glucantime^R) is equally well accepted for American leishmaniasis (Chulay *et al.*, 1988: McGrevy and Morsden, 1986). The recommended dose and schedule is summarized in table 3.

Aromatic Diamidines:

The search for alternatives to antimony therapy, led to the discovery of some aromatic diamidines, such as stilbamidine (Ashley *et al.*, 1942), hydroxystilbamidine (Kagan *et al.*, 1987), and pentamidine (Ashley *et al.*, 1946; Ashley and Harris , 1946; Fastier, 1962). In 1944, stilbamidine was used for treatment of kala-azar in Mediterranean region. It has several side effects, viz. lowering of blood pressure, respiratory disorder, neurotoxicity and trigeminal neuropathy

Table3 : Drugs in Use Against Visceral Leishmaniasis in Humans*

Drug	Dose schedule	Toxicity	Mode of action	Status in clinical medicine
Drug	Dose schedule	Toxicity	Mode of action	Status in clinical medicine
Pentostam (Sodium stibogluconate)	10 mg/kg of Sb^{+} for 7-10 days intramuscularly,repeat the dose twice or thrice with 10 days interval[b]	Arthralgia,myelgia,anorexia,nausea, vomiting, headache,epigastric discomfort, fever,weakness,dizziness,insomnia, nervous and renal damage,ECG changes	Inhibits phosphorylation of ADP to ATP citric-acid cycle. Also inhibits initial steps in glycolysis and certain enzymes of Krebs cycle and fatty acid oxidation	Drug of choice
Glucantime (Meglumine antimoniate)	17-28 mg/kg of Sb^{+} for 10-20 days intramuscularly,repeat the dose after 15 days if needed	Various side effects of Sb^{+} including erythemia,pruritis,rare death due to severe dysrhythmias e.g. ventricular fibrillation	Very much similar to sodium stibogluconate	Drug of choice
Pentamidine	4 mg/kg, 3 times(im) given alternately for 5-25 weeks[d]	Abdominal pain,weakness,nausea, vomiting,nephrotoxicity,liver malfunction, changes in carbohydrate metabolism,e.g. hypoglycemia and diabetes,occasional hypotension and collapes may also occur	Binds with DNA helix through electrostatic attraction between negative phospeate group and positive centre of amidine. Also damages mitochondria and kinetoplast-DNA core of both promastigotes and amastigotes. It may further inhibit RNA polymerase and biosynthesis of nucleic acids, proteins,phospholipids and polyamines	Second-line drug
Amophotericin B	Initial dose 5-10 mg/day is increased slowly until a dose level of 0.5-1 mg/kg is reached[c]	Causes serious nephrotoxicity. Other side effects include nausea anorexia,vomiting, fever,chills,anemia and local thrombophlebitis	Interacts with membrane cholesterol and its metabolites (e.g. ergosterol) in leishmania leading to increased permeability which causes loss of low molecular weight components such as glucose and amino acids	Second-line drug

Recommended by Wellcome Foundation, Ltd., Beckenham, U.K.

Recommended by W.H.O.

Recommended by Rhodia, S.A., Sao Paulo, Brazil.

(Napier *et al.*, 1942). Hydroxystilbamidine being stable and less toxic was preferred over stilbamidine (Sen Gupta, 1950; Snape, 1952).

Pentamidine:

Pentamidine is the drug of choice for treating Kala-azar patients, who have failed to respond to antimony therapy (Jha 1983). Pantamidine is less toxic than stilbamidine and can be administered intramuscularly. It is more effective against visceral leishmaniasis than cutaneous leishmaniasis and mucocutaneous leishmaniasis (Steck, 1974; Iyer, 1985). The recommended dose, mode of action and various side effects are summarized in table 3.

Antifungal antibiotics:

(I) Amphotericin-B:

Amphotericin B desoxycholate is a polyene antibiotic. It is effective against cutaneous and mucocutaneous leishmaniasis, (Sampio *et al.*, 1985). Amphotericin B is 400 times as potent as sodium stibogluconate in infected hamsters and monkeys. Amphotericin B is formulated as a colloidal suspension which is administered as a slow intravenous infusion. Its usefulness is limited by adverse reactions including anaphylaxis, thrombocytopaenia, flushing, generalized pain, convulsions, chills, fever, phlebitis, anaemia, anorexia, decreased renal tubular and glomerular function and hypokalaemia in about one-third of patients treated (Bryceson, 1978). It has therefore, never been considered a satisfactory first line drug for leishmaniasis and there is little evidence of its use in visceral leishmaniasis (Thakur, 1991: Mishra *et al.*, 1992). Amphotericin B tried at very low dose administration on alternate days, with a shorter schedule was found to be more effective with moderate toxicity (Mishra *et al.*, 1994; Thakur *et al.*, 1994; 1996).

Amphotericin B is still of considerable interest because of its mode of action. It binds to sterols in the plasma membrane, forming pores and leakage of ions. It binds preferentially to 24 substituted sterols such as ergosterol, which is the major cell membrane sterol of *Leishmania* parasite but not of mammalian cell membranes (Barman, 1991). To a lesser extent it binds to cholesterol in human membranes, and this leads to its toxicity. (Lianos-cuentas *et al.*, 1991). The major advancement has been the new formulations of amphotericin B with lipids (Pearson, *et al*, 1996).

Liposomal amphotericin B(AmBisome, Vestar, San, Dimas, CA, USA) was developed by incorporating amphotericin B into liposomes made of phosphatidylcholine, cholesterol, and disteroyl phosphatidyl glycerol. Pharmacokinetic data, indicates that AmBisome (Liposomal amphotericin B) is well suited for the treatment of visceral leishmaniasis. It has been used successfully in patient with visceral leishmaniasis unresponsive to standard treatment (Davidson *et al.*,1994;1996, Seamon *et al* 1995). Amphocil is a mixture of amphotericinB with cholesterol sulphate (a 1:1 molar ratio)

has shown to be effective against visceral leishmaniasis (Dietze *et al.*, 1993; 1995).

(ii) Ketoconazole:

This drug has been used to treat cutaneous and visceral leishmaniasis (Berman, 1988; Sunder *et al.*, 1990; Wali *et al.*, 1990). Like pentamidine and amphotericin B, Ketoconazole was also found effective in antimony resistant cases (Berman, 1982; Scott *et al.*, 1992; Wali *et al.*, 1990; Sunder *et al.*, 1990). The major side effects of the drug are vomiting, nausea and hepatic toxicity. Itraconazole is safer than ketoconazole and has a more favorable pharmacokinetic profile, being retained in skin for up to two weeks.

Other Antileishmanial Drugs

(I) Levamisole:

In the chronic forms of *L. tropica* infection, good therapeutic response by levamisole in clinical trial has been reported by Butler, (1978) and also against *L. mexicana* in mice (Grimaldi *et al.*, 1980; Rezai *et al.*, 1988).

(ii) Aminosidine :

Aminosidine (Paromomycin) is an antibiotic of amino-glycoside family. It is available as an injectable formulation given either by intramuscular injection or intravenous infusion. Aminosidine is highly effective against visceralizing species and Old World cutaneous leishmaniasis, but susceptibility varies in different leishmanial species of New World cutaneous leishmaniasis. Aminosidine is found to be highly active against antimony resistant strains. It is nearly six fold and more than 600 fold active than sodium stibogluconate against *L. mexicana* and *L. major*, respectively. Aminosidine is a safe, well tolerated effective 'first line' alternative to pentavalent antimony for the treatment of native and unresponsive visceral leishmaniasis (Olliaro and Bryceson, 1993).

Although chemotherapy has been available for many years but it is not sufficient to cure the patients of leishmaniasis. The drugs are fairly toxic, duration of treatment is undefined, mechanism of action is poorly understood and their success rate is variable and particularly poor in immunodepressed individuals most notably in patients with acquired immunodeficiency syndrome (AIDS). Visceral leishmaniasis (VL) has now been established as an HIV-associated infection. The immunopathological picture in VL/HIV infections is different. Patients with VL and HIV infections have defecting cell mediated immunity. They need prolonged treatment with antimonials and are liable to relapse. The recent epidemics of VL, parasite resistance to antimony, and the poor response in patients suffering from HIV infection has increased the urgency of new therapy or

drugs for leishmaniasis.

IMMUNOCHEMOTHERAPY:

The success of any chemotherapeutic regimen is often depend on the potential or latent immunological response by the infected host. Successful chemotherapy of leishmaniasis in human results in the generation of antigen-specific T-cells and delayed type hypersensitivity (DTH). When the patients have defective immune response, chemotherapy tends to be ineffective (Rizzi *et al.*, 1988).

The macrophage is an essential cell for *Leishmania donovani*, because it is the only cell in which the *Leishmania* species replicate during mammalian infection. Therefore, an alternative approach to therapy of visceral leishmaniasis would be to take the advantage of the host macrophages. Macrophages have been implicated in host defense against tumors and various intracellular and extracellular pathogens. The effective control of *Leishmania* infection in both mice and human has been shown to occur through cytokine induced macrophage activation (Green *et al.*, 1991; Murray, 1990; 1994a; 1994b).

Two new immunotherapeutic approaches in VL have been explored treatment with IFN-γ alone or treatment with IFN- γ plus conventional antileishmanial drugs. The appeal of the latter regimen stems from its potential to simultaneously attack intracellular *L. donovani* by different mechanisms: one utilizing direct toxicity to the protozoan with the parasitized macrophage (antimony) and the other involving effects on the macrophage itself (Murray, 1990;1994). The studies have demonstrated that IFN-γ enhances both *in vitro* and *in vivo* antileishmanial effects of sodium stibogluconate (Murray *et al.*, 1988). Recently a successful trials of combined therapy of IFN-γ and antimony has been carried out in Indian kala-azar patients (Sunder *et al.*, 1994; Shyam *et al.*, 1995). It was observed that patients who were earlier unresponsive to antimonials were found to be cured with this immunochemotherapeutic regimen. Successful clinical studies with GM-CSF have also been reported, although the efficacy of this cytokine is mostly related to the reversal of neutropenia (Badaro *et al.*, 1994; 1995).

IMMUNOTHERAPY:

Immunotherapy is comparatively better than chemotherapy. It directly induces cell mediated immune response. Various immunopotentiator, including *Bacillus calmette-Guerin* (BCG) (Fortier *et al.*, 1987), BCG plus promastigotes (castes *et al.*, 1989), levamisole (Rezai *et al.*, 1988),cyclosporin A (Bogdan *et al.*,1989), *C. parvum* (Hill,1987),and glucan (Cook *et al.*, 1980) have been used to modify the course of *Leishmania* infection. These immunopotentiators have an ability

to activate macrophages non-specifically. The complex immunopotentiators like BCG and *C. parvum* have been excluded from general human usage because of their undesirable side effects. The recombinant cytokines are more effective, more controlled and have negligible side effects against *Leishmania* infection. Several cytokines have been tested in models for intervention of visceral leishmaniasis, including IFN-γ (Murray *et al.*, 1988), IL-1 (Curry and Kaye, 1992), IL-2 (Murray *et al.*, 1993), TNF-α (Tumang *et al.*, 1994), GM-CSF (Murray *et al.*, 1995) and IL-12 (Murray and Hariprasad, 1995). Success has been variable and precise mode of action is difficult to determine in some cases. The effective cytokine therapy requires the presence of T-cells or other cytokines. The complexity of experimental cytokine therapy is particularly evident from recent studies on IL-12.The IL-12 can significantly reduce liver parasite burden and recipients of IL-12 indicate the presence of $CD4^+$ and $CD8^+$ T-cells, NK-cells and endogenous IL-2, TNFα and IFN-γ (Murray and Hariprasad, 1995). Therefore, number of workers have suggested that immunological intervention of disease in combination with chemotherapy is much better than chemotherapy or immunotherapy alone.

VACCINATION :

Vaccination against leishmaniasis has a long history (Alexander, 1988b). From as early as the 19th century, and as recently as 1990, living organisms have been used for vaccination (Peters *et al.*, 1990). Vaccination with *L. arabica* against *L. major* infection observed exacerbated lesion growth. Experimental studies using subcutaneous vaccination with heat-killed or radio-attenuated parasites have also often resulted in disease exacerbation following challenge of infective parasites (Liew *et al.*, 1985b). Thus, these studies were not useful as a vaccine in humans. After several years, Scientists prompted to try and use killed parasites, with or without adjuvant, as vaccines, or as an immunotherapeutic agents. These studies have shown some light in the progress of development of vaccine, which is not sufficient and still lot is to be done. In order to limit the development of a candidate vaccine, it should consists immunologically characterized, purified antigen or its derivatives. Studies have shown that apart from parasite membrane antigens, soluble non-membrane antigens can also induce protection (Handman and Mitchell, 1985; Russell and Alexander, 1988; Scott *et al.*, 1989). There are two types of vaccines, (I) 'first generation' vaccines are composed of heat killed or radio-attenuated parasites with or without adjuvant and (ii) the 'second generation' vaccines have different recombinant molecules, either parasite fractions or genetically constructed by removing virulent genes or bacteria carrying and expressing leishmanial genes which are avirulent. At present, the "first generation" vaccines are at various stages of Phase I (safety), II (reactivity) or III (efficacy) trials in humans. In contrast, 'second generation' vaccines are only in the preclinical state(Modabber, 1989; 1995; Kaye *et al*, 1995) (Table 4).

TABLE 4

Potential leishmaniasis vaccines currently being developed

Candidate vaccine	*Current development stage*	*Reference*
KILLED PARASITE WITH OR WITHOUT BCG	Clinical trials	
L. mexicana ± BCG, *L. braziliensis* + BCG	Field efficacy, immune responses	Convit *et al.* (1987), Castes *et al.* (1994)
L. major ± BCG*	Safety, immunogenecity (Phase I–II)	Bahar *et al.* (1993)
Mixed *Leishmania* strains	Efficacy	See Antunes *et al.* (198
L. amazonensis	Preparation, Phase I–II	K. Marzochi (unpubl. obs.)
	Immune responses	Mendonca *et al.* (1995)
LIVE VACCINES	Pre-clinical development	
Recombinant *Leishmania* (DHFR/TS⁻)	Development and animal testing	Cruz *et al.* (1991), R. G. Titus *et al.* (1995)
Recombinant bacteria		
Salmonella–gp63	In-vivo immunization (mice, dogs)	Yang *et al.* (1990)
BCG–gp63	Preparation and mouse vaccination	Connell *et al.* (1993)
Recombinant virus	Preparation and animal testing	McMahon-Pratt *et al.* (1993)
Vaccinia-gp46	Primate studies	G. Grimaldi (unpubl. obs.)
DEFINED SUBUNITS	Pre-clinical development	
r-gp63	Animal studies	Russo *et al.* (1991)
LelF	Mice and in-vitro human cells	Skeiky *et al.* (1994)
LACK	Mice and in-vitro human cells	Mougneau *et al.* (199 Rafati *et al.* (unpubl. obs.)
OTHERS		
dp70-72	Gene cloned and protein expressed	Rachamin *et al.* (199
Synthetic peptides	Animal studies	Jardim *et al.* (1990)

*Field efficacy trials are underway in Iran, Pakistan and Sudan.

The 'second generation' vaccine candidates can be divided into three categories: (I) live vaccine (ii) defined subunits and (iii) crude fractions. It is possible to produce stable mutants which, at least, would cause an abortive infection, if inoculated into human (Cruz *et al.*, 1991). An other attempt to develop an oral vaccine against leishmaniasis, a *Salmonella typhimurium* that expresses the *L. major*, promastigote surface protein gp63 has been constructed. It is found that the construct is stable, capable of expressing the protein and could induce T_H1 type response in mice (Yang *et al.*, 1990). Another important development is the construction of recombinant BCG carrying gp63, which has shown to protect mice against *L. maxicana* infection (Connel *et al.*, 1993).

Defined recombinant subunit and synthetic vaccines have been shown to induce protection in experimental animals. Various recombinant proteins or synthetic peptides, like r-gp63 (gp63 expressed in *E. coli*) or various synthetic peptides of gp63 have represented T-cell epitopes and have also induced partial protection (Russo *et al.*, 1991; Jardim *et al.*, 1991). Another recombinant protein of *L. braziliensis* (LeIF), stimulates human T_H1-type cells and induces IL-12 production by normal peripheral monocytes, *in vitro*. A protein of *L. major*, *Leishmania* homologue of receptors for activated C kinase (LACK) which could activate protein kinase C, was used as protective T-cell clone against infection (Rivas *et al.*, 1991; Mougneau *et al.*, 1994). Russell and Alexander (1988) reconstituted crude Ag or purified Ag (gp63) into liposomes and then inoculated into CBA/Ca or BALB/c mice, both by subcutaneous and intraperitoneal routes. They observed complete protection in CBA/Ca mice,when administered by both routes and poor protection in BALB/c mice.

Handman and Mitchell (1985) reported successful vaccination of BALB/c mice with purified *L. major* LPG. Interestingly, soluble LPG that lacked the phosphatidylinositol anchor induced a disease exacerbation. Similar protection against *L. mexicana* in CBA/Ca mice is also reported with homologous LPG, which is constituted into liposomes (Russell and Alexander, 1988). Purified LPG from *L. major* reconstituted into liposomes with or without *C. Parvum* when inoculated into BALB/c mice prior to infection resulted in survival of animals (McConville *et al.*, 1987). The protective efficacy of LPG has been speculated to be due to some peptides/proteins associated with LPG. There are several proteins which are tightly associated with LPG, such as the B protein and KMP-11. These proteins are highly immunogenic in nature which lack NH_2 terminal signal peptides (Jardim *et al.*, 1991; 1995; Flinn *et al.*, 1994). These peptides are responsible for the T-cell response to LPG (Jardim *et al.*, 1991; Moll *et al.*, 1989). Jaffe and colleagues have isolated and purified a protein which is associated from *L. donovani* LPG molecules. This protein protect mice against cutaneous as well as, visceral leishmaniasis (Rachamim and Jaffe,1993).

Although recent vaccine literature is available in abundence and many antigens have been studied, the advantages and disadvantages of each approach for vaccine development have been discussed above but molecular vaccine development are open to question.

CHAPTER 2

LIPOPHOSHOGLYCAN:

THE STORY SO FAR IN LEISHMANIASIS

The protozoan are the most diverse and amongst the most ancient group of organisms in the eukaryotic kingdom (Sogin *et al.*, 1989). Many of their members are parasitic, like, those belonging to the family Trypanosomatidae (African trypanosomes, *Trypanosoma cruzi, Leishmania* spp.) and the genera *Plasmodium, Eimeria, babesia, Theileria, Toxoplasma* and *Entamoeba*, and are the cause of important diseases in humans and their domestic liver stock. These parasites express cell surface glycoconjugates. Glycoconjugates on the cell surface of these organisms play a crucial role in determining parasite survival and infectivity (McConville and Ferguson, 1993). Such as, *Leishmania* spp., growing in culture produce a polysaccharide or glycoconjugate, called the Excreted Factor (EF) (Schnur *et al.*, 1972). It occurs on the parasite cell surface as well as in the culture medium and is the major antigenic glycoconjugate released from the surface of leishmanial parasite (Schnur *et al.*, 1977; Slutzky *et al.*, 1979; El-on *et al.*, 1979). Excreted factor is immunologically active substance and species-specific (Schnur *et al.*, 1972). EFs are responsible for parasite infection for host macrophages (Handman and Greenblatt, 1977). Excreted factors are also produced by intracellular amastigotes (Schnur *et al.*, 1972). EFs from *Leishmania tropica* and *Leishmania donovani* share a common carrier protein, identified as rabbit serum albumin (Slutzky *et al.*, 1979). Excreted factors contains about 45.6% glucose, 39.9% galactose, 14.2% mannose and a trace amount of glucosamine (Slutzky and Greenblatt, 1979). The molecular weight of the excreted factor ranged between 15-30 kDa.

Excreted factor is organized into three categories: the first category is called cell associated, i.e. it forms very tight complex with albumin in the medium and can be dissociated by treatment with TCA into two fragments, one having a molecular weight of about 61,000 (without anti leishmania activity) and the other having, a molecular weight of about 18,000 (with no anti-rabbit activity) (Slutzky *et al.*, 1979). This form of excreted factor has a property to react with certain lectins and also show positive reaction with periodic acid-schiff's stain and is called lipophosphoglycan. The second category is the soluble secreted form of excreted factor which are isolated and purified from promastigotes. They are highly negatively charged polysaccharide. These appear to be immunologically identical substance, as secreted acid phosphatase by *L. donovani*, *L. tropica* and *L. mexicana* (Bates *et al.*, 1990; Jaffe *et al.*, 1989; 1990, Ilg *et al.*, 1991). The third category of excreted factor is an extracellular phosphoglycan (exPG), which is purified from culture medium of *L. donovani* (Greis *et al.*, 1992). Their structural characterization indicates that they consist of the following structure: (CAP) [PO4-6Gal (β1-->4) man α1)] 10-11. The cap contains one of the several small neutral oligosaccharides, most abundant is the branched trisaccharide Gal (β1-->4) [Man (α 1-2)] Man (α1). ExPG is identical to LPG except that it lacks the lipid anchor and the phosphosaccharide core, with lesser number of repeating units as compared to LPG (Fig. 5). The

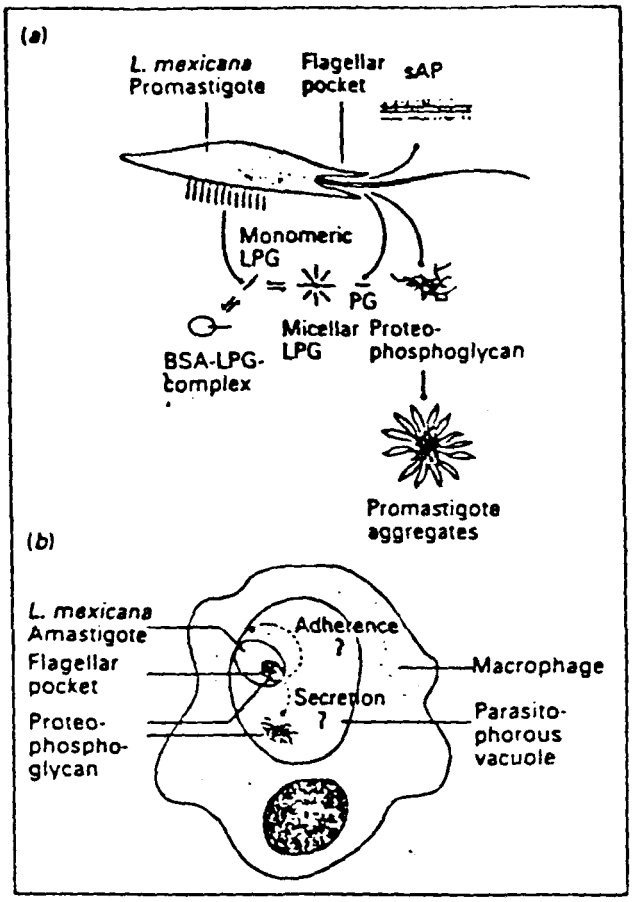


Figure 5: Summary of secretory products from promastigotes (a) and amastigotes of *L. mexicana* (b). (Ferguson *et al.* 1994).

leishmanial excreted factor contains terminal non reducing galactose residues (Slutzky and Greenblatt, 1982). This galactose residue is found to be immunodominant sugar in these excreted factor.

Importance of cell-surface Glycoconjugates:

1. The cell surface glycoconjugates play a key role in the survival of the *Leishmania* parasite throughout its life cycle. It protects the parasite in the gut of the sandfly where the organism could be susceptible to digestive enzymes.
2. It protects the parasite in the bloodstream of the vertebrate host where they transiently exist and would be exposed to the lytic complement pathway.
3. In the phagolysosome of the host macrophages where the parasite would be vulnerable to hydrolytic enzymes and the microbicidal oxidative burst.
4. It is possible that Excreted factors promote immunity to leishmanial infection, such a general EF could provide a broad spectrum of protection, whereas the species-specific EF would only protect against a single species (Slutzky *et al.*, 1979)
5. Interaction of excreted factor with macrophages may be helpful.

A number of functions have been suggested for excreted factor; it inhibits the activity of lysosomal β-galactosidase of macrophages (El-On *et al.*, 1980). The excreted factor may be either a substrate for mammalian lysosomal β-galactosidase or a competitive inhibitor of the enzyme. The excreted factors isolated from the culture fluid in which *L. tropica* and *L. donovani* were grown precipitated C-reactive protein. (Pritchard *et al.*, 1985). It has also been demonstrated that leishmanial excreted factor binds to C-reactive protein which may have a significance in host defense against these organisms.

LIPOPHOSPHOGLYCAN (LPG):

The lipophosphoglycan is a major cell surface glycoconjugate of the *Leishmania* parasites. It is expressed preferentially during the latter part of the logarithmic growth phase and during stationary phase, and is finally released into the culture medium (King *et al.*, 1987). It is observed that the presence of albumin in the medium enhances the rate of release of LPG from cells. It is postulated that the lipid moiety of LPG interacts with hydrophobic binding site on albumin (King *et al.*, 1987) and this interaction facilitates the released of LPG into medium. It is a heterogeneous lipid containing polysaccharide molecule (Turco and Descoteaux, 1992). The molecular weight of acidic glycoconjugate is found to be in the range of 15,000-30,000 (Turco *et al.*, 1984) and later it was found to be between 10-20 kDa of LPG (King *et al.*, 1987). LPG is thought to play an important role in the biology of the parasite due to its surface location, its developmental regulation during the life cycle and the reduced virulence of the LPG deficient organisms (Elhay *et al*, 1990;

McConville *et al.*, 1992). A variety of functions and activities of LPG in mammalian host have been experimentally demonstrated or suggested. These include involvement in attachment and detachment in sandfly midgut, attachment and entry of promastigotes into mammalian macrophages, protection of parasite within the phagolysosomal compartment, inhibitor of PKC, inhibitor of oxidative burst and scavenging of oxygen metabolites, inhibitor of chemotactic activity and as a recognition molecule for the T-lymphocyte-dependent immune responses characteristic of leishmaniasis (Handman and Goding, 1985; Tolson *et al.*, 1990; Turco, 1990; 1993; Moll *et al.*, 1989; Descoteaux and Turco, 1992; Pimenta *et al.*, 1992; 1994; Sacks *et al.*, 1995; Sacks and Turco, 1995; Panaro *et al.*, 1995). In the light of these functions, the knowledge of biological actions of LPG may be useful in order to prepare a vaccine against human leishmaniasis.

STRUCTURE OF LIPOPHOSPHOGLYCAN:

Lipophosphoglycan of promastigotes:

The promastigotes of all *Leishmania* parasites produced LPG (King *et al.*, 1987; Pimenta and Sacks, 1991). The general structure of LPG molecule consists of four domains (Figure 6):

a) a phosphatidylinositol lipid anchor,
b) a phosphosaccharide core,
c) a repeating phosphorylated disaccharide region, and
d) a small oligosaccharide cap structure.

The lipid anchor of LPG:

The polysaccharide portion of LPG is anchored by the unusual phosholipid derivative viz. 1-O-alkyl-2-lyso-phosphatidyl(myo) inositol (Orlandi *et al.*, 1987; McConville *et al.*, 1987, Ilg Thomas *et al.* 1992). The aliphatic chain of LPG in *L. major*, *L. donovani* and *L. mexicana* is attached by ether linkage to carbon-1 of the glycerol backbone. It can be either a C_{24} or C_{26} saturated, unbranched hydrocarbon (McConville *et al.*, 1987). The molar ratio in alkyl lyso-PI anchor chains is found to be 73% and 21% for C_{24} and C_{26}, respectively.

The Phosphosaccharide Core of LPG:

The Phosphosaccharide core of LPG is attached to the inositol of the lipid anchor. The glycan core consists of an unacetylated glucosamine, two mannose, a galactose -6- phosphate, a galactopyranose, and a galactofuranose (Turco *et al.*, 1989; McConville *et al.*, 1990, Ilg Thomas *et al.*, 1992). LPG possesses the Man (α1–>4 Glc N (α 1–>6) myoinositol-1-PO_4 motif. The LPG core of *L. donovani* (Thomas *et al.*,1992) and *L. mexicana* (Ilg Thomas *et al.*, 1992) possess a galactosyl- α 1- phosphate attached via phosphodiester linkage to the C_6 hydroxyl of the proximal

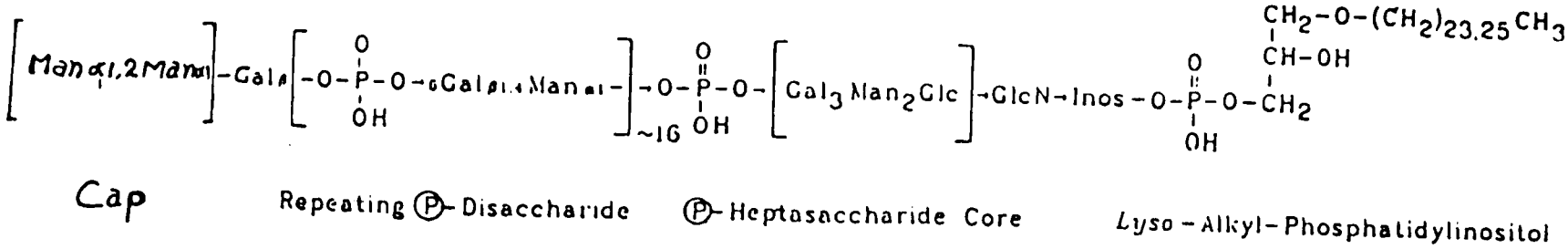


Figure 6: Structure of the *L. donovani* LPG (Turco, 1990).

mannose residue. The *L. mexicana* LPG resembles that of *L. donovani* LPG having phosphosaccharide core in which Man 2 is always substituted with Glcp $\alpha 1PO_4$. A substantial percentage of *L. major* LPG contains an identical glucosyl-α1-phosphate substitution. The Gal (α1-3) Gal unit of the phosphosaccharide core has been found to be the epitope for circulating antibodies in patients with leishmaniasis (Avila and Garcia , 1988; Avila and Towbin, 1988; Towbin *et al.*, 1987).

The repeating Units of LPG:

All LPG molecules contain multiple units of phosphorylated disaccharide repeat of PO_4-6Gal (β1–>4)Man (α1) (Ilg Thomas *et al.*, 1990, Turco *et al.*, 1987). These phosphorylated disaccharide repeat units are attached by α-glycosidic linkage. The disaccharide repeat units of *L. donovani* LPG has no substitutions in their backbone sequence whereas repeating units of *L. mexicana* LPG has approximately 30% of its galactose residues substituted at the C_3 hydroxyl with glucose (Glc) residues (Ilg *et al.*, 1992). The repeating units of the *L. major* LPG are the most complex, as more than 85% of the galactose residues are further substituted with small saccharide side chains containing one to four residues of galactose, glucose, or the pentose arabinose (McConville *et al.*, 1990). Thus, the extra Glc of *L. mexicana* and the extra Gal, Glc and Ara of *L. major* are always linked to the 3-position of the Gal residue of the phosphosaccharides repeat units (Thomas et al., 1992). It has been demonstrated that the number of repeating units per LPG molecule directly depends on the growth stage of promastigote. The average number of repeat units/molecule reported for different species are: 16 for *L. donovani*, 20 for *L. mexicana* and 14 for procyclic, 30 for metacyclic, 36 for amastigotes of *L. major*. (Kelleher *et al.*, 1994).

The Cap oligosaccharide of LPG:

The LPG molecule is terminated by the nonreducing neutral oligosaccharides or molecules like galactose or mannose. Although the LPG of *L. major* possesses the most complicated series of repeating units but it is capped with the simplest structure, consisting exclusively of the disaccharide Man (α1-2) Man (α1) (McConville *et al.*, 1990). The most abundant terminal oligosaccharide of *L. donovani* and *L. mexicana* LPG are the branched trisaccharide Gal (β1-4) [man (α 1-2)] Man (α 1) (Thomas *et al.*, 1992; Ilg *et al.*, 1992). In the *L. major* LPG, the Cap structure is predominantly Man α1-2-Man, whereas in *L. mexicana* and *L. donovani* LPG, the Cap structures are mainly Man α 1--> 2Man, Man α1-->2Man α1-->2Man, and Man α1--> 2[Gal β1-->4] Man. (Thomas *et al.*,1992).

The ratio of the oligosaccharide caps Man α1-->2 Man, Man α 1-->2[Gal β1-->4]Man, Man α1-->2 Man α 1-->2 Man, Man α 1-->2 Man α 1-->2 [Gal β1-->4] Man and Gal β1-->4 Man

is found to be 20:52:6:5:16. It is also observed that the ratio of cap species to total repeat units are found to be (1:12 and 1:25 for procyclic and metacyclic LPG respectively (McConville *et al.*, 1992).

The overall structure of LPGs isolated from *L. donovani* (Thomas *et al.*, 1992), *L. major* (McConville *et al.*, 1990) and *L. mexicana* (Ilg *et al.*, 1992) promastigotes are similar, with a linear arrangement of caps, repeating units, glucosylated core and lyso-alkylglycerol lipid moiety (Figure 7). The most striking difference in LPG structure between the *Leishmania* species lies in the phosphorylated oligosaccharide repeats. The *L. donovani* LPG possesses simplest, phosphorylated oligosaccharide repeat unit, where as in *L. maxicana* LPG, about one in five of the repeats is a trisaccharide with the structure Glc β–>3[PO$_4$6]Gal β1—>4 Man α1- and *L. major* LPG is the most complex structure (McConville *et al.*, 1987, 1990). The *L. major* phosphorylated disaccharide repeats are branched, with more than 85% substitution at the 3C position of Gal with di, tri and tetrasaccharide (McConville *et al.*, 1990; 1992).

Structural analysis of LPG from several species of *Leishmania* have shown complete conservation of the lipid anchor, extensive conservation of the phosphosaccharide core, variability of sugar composition and sequence in the repeating phosphorylated saccharide units and the cap structure (Ilg *et al.*, 1992; McConville *et al.*, 1990; Orlandi *et al.*,1987; Thomas *et al.*, 1992). The three-dimensional solution structure of the repeating PO$_4$6 Gal (β1–>4)Man α1-disaccharide unit of LPG derived from *L. donovani* has recently been determined. (Homans *et al.*, 1992). The molecular modeling study showed that each of the stable conformers of the Man (α1)PO$_4$6Gal linkage exists in a different configuration within the same LPG molecule. The torsional oscillations allow the LPG molecule to contract or expand in a manner reminiscent of a slinky spring, resulting in a molecule whose length can range from 90A°(fully contracted) to 160A°(fully expanded), assuming an average of 16 repeat unit (Homans *et al.*, 1992).

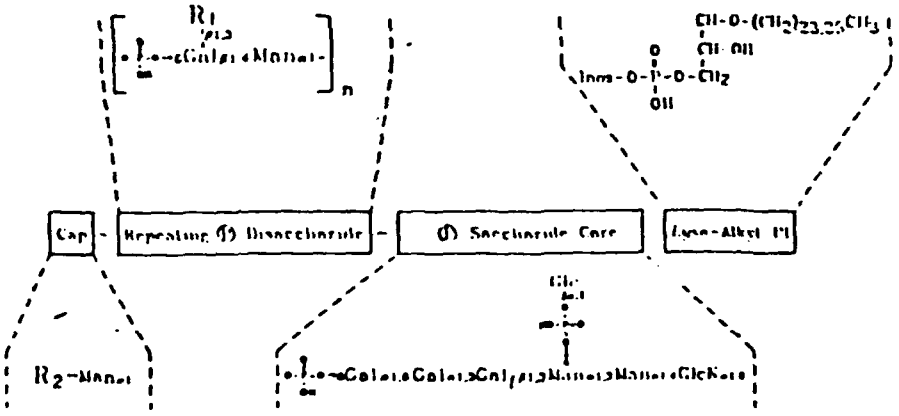
DEVELOPMENTAL MODIFICATION OF LPG METACYCLOGENESIS:

The most significant property of LPG is its structural modification accompanying the process of metacyclogenesis (Sacks *et al.*, 1985; 1987). Structural comparison of LPG isolated from logarithmic and stationary phase promastigotes of *L. major* revealed that lyso-1-O-alkylphosphatidylinositol lipid anchor, and the phosphosaccharide core are conserved (Sacks *et al.*, 1990) Figure 8. The most striking changes that occur during metacyclogenesis are as follows:

(I) An approximate doubling in the average number of repeat units per molecule .

(II) Decrease in the relative abundance of side chains of βGal or Gal β1-3Gal β1 and a corresponding increase in repeat units with either no side chains or with side chains of Arap α1-2 Gal β.

(III) A decrease in the frequency with which the glycolipid anchor is substituted with a single glucose



	R_1	R_2
L donovani (n_{avg}=16)	= H	= $Man_{\alpha1-2}$-
		= $Gal_{\alpha1-}$-
		= $Man_{\alpha1-2}[Gal_{\alpha1-}]$-
		= $Man_{\alpha1-2}Man_{\alpha1-2}$-
		= $Man_{\alpha1-2}Man_{\alpha1-2}[Gal_{\alpha1-}]$-
L major (n_{avg}=27)	= H	= $Man_{\alpha1-2}$-
	= Gal	
	= $Ara_{\alpha1-3}Gal$-	
	= $Gal_{\beta1-3}Gal$-	
	= $Glc_{\beta1-3}Gal$-	
	= $Ara_{\alpha1-3}Gal_{\beta1-3}Gal$-	
	= $Gal_{\beta1-3}Gal_{\beta1-3}Gal$-	
	= $Ara_{\alpha1-3}Gal_{\beta1-3}Gal_{\beta1-3}Gal$-	
L mexicana (n_{avg}=16)	= H	= $Man_{\alpha1-2}$-
	= Glc	= $Man_{\alpha1-2}Man_{\alpha1-2}$-
		= $Man_{\alpha1-2}[Gal_{\alpha1-}]$-

Figure 7: Structure of LPG from three promastigotes species of *Leishmania* grown in log to late log phase of growth (Turco and Descoteaux, 1992).

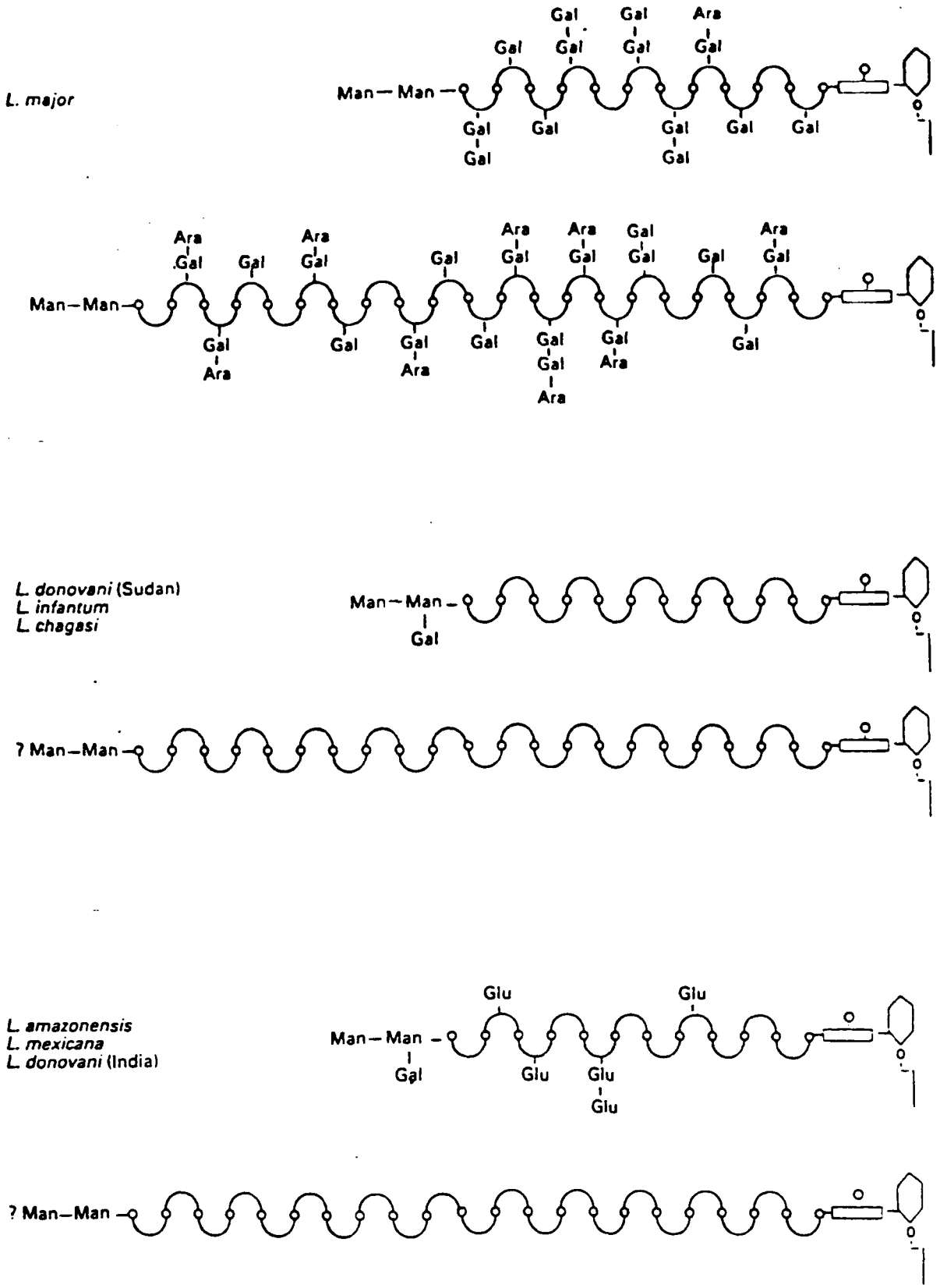


Figure 8: Schematic diagram of the stage and species-specific polymorphisms of LPG. The phos-gal-man backbone repeats are increased in number for metacyclics and are represented with or without variable side-chain substitutions. The structures shown for *L. major* are modified from McConville *et al.* (1992). The procyclic and metacyclic LPGs represented for the other strains are based on preliminary data. (Ferguson *et al.*, 1994).

α-1 phosphate residue.

The surface metacyclic promastigote contains densely packed filamentous structures, which are ~20 nm in thickness. In contrast, the logarithmically grown, non infectious nectomonad promastigotes have a thin glyocalyx which is 10 nm in thickness. This variability is due to the differential expression in the LPG in these two stages. A striking difference in the repeating phosphorylated disaccharide composition in the two stages of LPG from *L. major* has been observed (Sacks *et al.*, 1985; 1990). The repeat units of LPG obtained from logarithmically *L. major* and *L. donovani* contain terminal galactose residue, whereas, LPG in metacyclic grown form possess Arabinose and glucose residues. The galactose residues are responsible for the agglutinability with peanut agglutinin, while in metacyclic promastigotes, the repeat units terminate predominantly with Ara and to a lesser extent with β-glc residues, which are not ligands for the lectin (Sacks *et al.*, 1990; Turco *et al.*, 1987; Volf *et al.*, 1994; McConville *et al.*, 1991; 1992). However, the terminal galactose residue present in the cap oligosaccharide of LPG isolated from noninfectious parasites is absent in metacyclic LPG. Such changes in LPG structure may have profound implications on its functions and suggest that it may have an important role in the regulation of the life cycle of *Leishmania* parasites. Therefore, the reduction in terminally exposed galactose residues controls the stage-specific adhesion of developing promastigotes to the sandfly midgut (Pimenta *et al.*, 1992; Sacks and Turco, 1995). *In vitro* binding of LPG to midgut could be completely inhibited with procyclic LPG or with purified repeat units containing side chains terminating in galactose, but not with metacyclic LPG or the metacyclic specific repeat units with arabinose - capped side chains.

Recent studies have revealed an identical modification of the LPG during *in vivo* metacyclogenesis. (Davies *et al.*, 1990; Lang *et al.*, 1991; Saraiva *et al.*, 1995). The Procyclic LPG detected by day 3 on a small proportion (18%) of procyclic and nectomonad is formed in the abdominal midgut. By day 5, virtually all promastigotes in the midgut expressed high levels (90-100%) of procyclic LPG on their surface, and a few begin to exhibit internal staining for metacyclic LPG. In contrast, 2% metacyclic LPG is present on day 5, 40% on day 7 and 60-70% on day 10, in the midgut to *P. papatasi* (Saraiva *et al.*, 1995). Even at 15 days, roughly 30-40% parasites in the gut have not differentiated to metacyclic form. However, complete metacyclogenesis is rarely achieved in culture (da Silva and Sacks, 1987).

The developmental modification of LPG may be important in the binding of promastigotes to receptors in the sandfly midgut as well as on human macrophages and in increasing the resistance of metacyclic promastigotes to complement mediated lysis (McConville *et al.*, 1992; Camara *et al.*, 1995). The expression of LPG on newly transformed promastigotes is found to be delayed and it could not be detected upto 3 days. It is possible that delay is a consequence of time required for synthesis and surface expression of LPG after transformation. Alternatively, since the

expression of LPG coincides with the morphogenesis of nectomonad forms, it is possible that its biosynthesis does not occur during the transformation of amastigotes to procyclic promastigotes. Therefore, it has been suggested that delayed expression of LPG on developing promastigotes would argue against the role of LPG in protecting the parasites from the proteolytic enzymes in the midgut of the sandfly vector (Dilon andLane, 1993; Saraiva *et al.*, 1995).

Species Specific LPG Polymorphisms and Vector Competence:

The sandfly alimentary tract can pose a number of potential barriers to the complete development of transmissible infection in the vector. The death and loss of *Leishmania* pomastigotes occur in an inappropriate vector, due to presence of proteolytic enzymes which are secreted for the digestion of the infective meal (Schlein and Romano, 1986; Borovisky and Schlein, 1987). Proteolytic activity could be modified by released glycoconjugate. Since shed glycoconjugate contains large amounts of LPG, hence LPG polymorphisms might be driven by species variations and specificities of digestive enzymes in the sandfly gut.

The majority of experiments involving infection of sandflies with parasite strains which they do not normally transmit in nature have been carried out with *P. papatasi.* and a closely related species, *P. dubosqui,* are the natural vectors of *L. major* and do not transmit any other species (Sacks *et al.*, 1994). It has been shown that *P. Papatasi* supports the full growth and development of *L. major* in high frequency whereas this is unsuitable for the development of *L. donovani, L. infantum, L. tropica, L. braziliensis* and *L. amazonensis* (Adler and Theodor, 1927; Heyneman, 1963). *P. Sergenti,* a natural vector of *L. tropica* is able to support experimental infection with *L. tropica* but not of *L. infantum* and *L. major.*

It is possible that LPG polymorphism might be driven by heterogeneity in parasite recognition sites expressed on midgut epithelial cells of different sandfly species. It is observed that procyclic promastigote of *L. major* is attached only to the epithelium of midgut of its vector *P. papatasi,* while promastigotes of other species fail to attach to epithelium midgut of this vector. Identical differences in binding are observed after using LPG molecule from each of the species.

The ability of *L. major* LPG to mediate procyclic attachment is probably determined by a unique aspect of its structure that the multiple β-1-3 side chain-linked terminal galactose residues per molecule are responsible for binding. The LPG molecules of the other species are also examined, in these terminally exposed galactose residues are either absent entirely, or restricted to the single neutral capping oligosaccharide expressing the branched trisaccharide, Gal β1->4 (Man α 1->2) Man. This suggests that the putative receptors on *P. papatasi* midguts are either highly specific for β1-3 linked galactose residues, or as is more likely the case, dependent on recognition of multiple ligands for stable binding to occur. Therefore, *P. papatasi* may lack receptors for LPG of other *Leishmania*

spp. These LPGs might mediate significant binding to midguts of their natural or at least permissive vector. Similarly, procyclic promastigotes or purified procyclic LPG of *L. donovani* show a significant and comparable binding to the *P. argentipes* midguts, which is natural vector of Indian *L. donovani* infection. Other Species, *L. major, L. amazonensis* or the LPGs of these species show comparatively low binding to midgut of *P. argentipes*. Thus, *P. argentipes* midgut possess a receptor, lacking in *P. papatasi*, for relatively conserved oligosaccharide on LPG. The observed significant differences in LPG mediated binding argue that the alimentary tracts of at least some Phlebotomine vectors are functionally diverse, and midgut therefore, provide the evolutionary drive for LPG structural polymorphisms.

LIPOPHOSPHOGLYCAN OF AMASTIGOTES:

All the detail information regarding the structure of LPG has been obtained from *Leishmania* promastigotes. The presence of LPG has also been examined in the amastigotes of *L. donovani, L. major* and *L. mexicana* but distinguish features have been obtained. The *L. donovani* amastigotes apparently can not synthesize LPG, as at least a 10^4 fold down-regulation in LPG was found corresponding to less than 100 molecules/cell and *L. mexicana* 10^3 fold down-regulation summarised in table 5. In an another report, it has been shown that *L. major* amastigotes express LPG that is both biochemically and antigenically distinct from that of promastigotes (Turco *et al.*, 1991; Moody *et al.*, 1991; Glaser *et al.*, 1991). Like promastigote LPG, the amastigotes LPG also consists of mainly three domain: The lipid anchor, hexasaccharide core and phosphoglycan. The structure of the anchor, the core and the phosphoglycan were determined by monosaccharide and linkage analysis, fast atom-bombardment mass spectrometry, one dimensional ^{1}H NMR spectroscopy, and exoglycosidases microsequencing (Moody *et al.*, 1993). Amastigotes LPG consists of galactose, glucose, mannose, glucosamine and inositol monosaccharides in a molar ratio of 51:30:24:1:1. Amastigotes LPG lacks arabinose. The lipid anchor consists four types of alkyl chain with length of 24:0, 22:0, 20: and 26:0 carbon atoms in the molar ratio 68:18:8:6. The phosphate is present at 4% w/w of total carbohydrates content (Moody *et al.*, 1991). It is found that amastigotes LPG belongs to a polydisperse family of molecules, showing a band spanning region of Mr 55-100 kDa (Glaser *et al.*, 1991; Moody *et al.*, 1993). Therefore, the LPG of amastigotes possess a larger molecular weight than promastigotes, while expressing at a lower copy number/cell. The average number of repeat units per molecule is 36 (McConville *et al.*, 1992; Moody *et al.*, 1993). Amastigotes LPG consists of PO_4 6Galβ1–4Man α 1 repeats which are either unsubstituted 70% or substituted 30% at the 3-C position of the Gal residues with oligosaccharide side chain containing primarily of Gal and Glc (Moody *et al.*, 1993).

TABLE 5:

Determination of lipophosphoglycan in *Leishmania*

Leishmania spp.	Promastigotes (molecules cell $^{-1}$)		Amastigotes (molecules cell $^{-1}$)
	Log phase	Stat. Phase	
L. mexicana	1×10^6	$1.3\text{-}2 \times 10^6$	$<10^3$
L. major	1.4×10^6	$1.1 - 5 \times 10^6$	2×10^3
L. donovani	3.3×10^6	$2.5 - 5.6 \times 10^6$	$<10^3$

LPG from amastigote and promastigote forms of *L. major* have many commo characteristics, such as:

i) The amastigotes LPG was extracted by same protocol as the LPG from promastigotes, indicating the similar solubility properties.

ii) Amastigotes LPG is susceptible both to nitrous acid deamination and PI-PLC treatment.

iii) The PI-PLC released PG from amastigotes LPG was found to have a similar structure to the promastigotes counterparts.

iv) The amastigotes PG contains phosphate and was susceptible to mild acid hydrolysis.

During amastigote to promastigote transformation, the amastigote specific form of LPG disappears after subculture for 48hr, whereas, during promastigote to amastigote transformation, the amastigote-specific form of LPG was detected after about 12 hr (Glaser *et al.*, 1991) The quantities of the *L. major* amastigotes version of LPG appear to be much less than the promastigote counterpart. The high molecular weight phosphoglycan appears to be located in the lumen of the flagellar pocket of amastigotes as seen in the mouse lesion and may be secreted from there into the lumen of the parasitophorous vacuole of the parasitized macrophages. LPG was undetectable in lysates of either amastigotes or infected macrophages (Bahr *et al.*, 1993). In both *L. mexicana* and *L. donovani* amastigotes as compare to *L. major* (McConville and Blackwell, 1991), the expression of LPG is down regulated at least 1000 fold. In agreement with several recent studies (Turco and Sacks, 1991; Glaser *et al.*, 1991) small amounts of LPG about 2 x 10^3 molecules/cell are detectable in *L. major* amastigotes (Bahr *et al.*, 1993). Finally, the amastigote surface is devoid of LPG as judged by immunofluorescence and immuno-electron microscopy in *L. mexicana* (Bahr *et al.*, 1993), while it was detected on the cell surface of *L. major* (Kellehere *et al.*, 1995). Functionally, amastigotes LPG is different than promastigotes LPG due to low expression of LPG molecules/cell. It play a protective role in amastigotes against macrophage lysosomal enzymes (Bahr *et al.*, 1993).

BIOSYNTHESIS OF LPG:

LPG is an essential molecule for the survival of *Leishmania* parasites as it progresses through out the various phases of its life cycle. The biosynthesis of LPG is as follows:

(1) Synthesis of the PI lipid anchor region:

Although LPG posses a lyso-1-O-alkyl-PI anchor, its synthesis may involve formation of 1-O alkyl-2-acyl-PI precursor. *Leishmania* parasites possess significant amount of 1-O-alkyl-2-acyl phospholipids, but not lyso-alkyl phospholipids (Wassef *et al.*, 1985). The lyso-alkylphospholipids are known to be cytotoxic for *Leishmania* (Acterburg and Gercken, 1987) as well as other eukaryotic cells (Hoffman *et al.*, 1984). On the basis of other eukaryotic system, it has been suggested that an ether phospholipid synthesis might be initiated by acylation of C_3 of glycolytic

intermediate, such as dihydroxyacetonephosphate (DHAP). A key enzyme, believed to be involved in ether lipid synthesis is dihydroxy acetone phosphate acyltransferase which has been reported in *Leishmania* glycosomes (peroxisome)(Hart and Opperdoes, 1984). The acyl group may then be replaced with a fatty alcohol. *Leishmania* parasite do incorporate fatty alcohols in ether phospholipids (Herrman and Gercken, 1980). The resultant alkyl dihydroxyacetonephosphate might then be reduced with NADPH and acylated at the Sn-2 position forming 1-O-alkyl-2-acyl phosphatidic acid. The latter may activate the CDP-derivative by CTP and then condensed with myoinositol to form 1-O-alkyl-2-acyl-PI.

(II) Assembly of the Core-PI region:

The pathway of core-PI synthesis can be derived from the recently elucidated structures of the GIPLs isolated from *Leishmania* spp. (McConville and Bacic, 1989; 1990; McConville and Blackwell, 1991; McConville *et al.*, 1990) and from details of GPI anchor assembly in African *trypanosomes* (Daeing *et al.*, 1990; Menon *et al.*, 1990).

In *Leishmania* species, addition of the first mannose residue to Glc-N-PI presumably would yield Man(α 1-->4) Glc N-PI, a precursor to LPG. The addition of the second mannose residue is at a branch point in the biosynthetic pathway of leishmanial GPI anchors. In the synthesis of the GPI anchor of gp63 (Schneider *et al.*, 1990) the second mannose would form Man (α 1--> 6) Man (α 1-->4) Glc N-PI, whereas in LPG, it would yield Man (α 1-->3) Man (α 1-->4) Glc-NPI. In LPG biosynthesis, three galactose residues then would be added, one of which is the galactofuranose.

(III) Polymerization of repeating units:

An *in vitro* membrane system from *L. donovani* capable of synthesizing LPG repeating units has been shown (Carver and Turco, 1991). The galactose and mannose residues of the LPG repeating units of *L. donovani* are believed to be added from their respective nucleotide-sugar donors sequentially and directly to LPG (Figure 9). The mannosyl-phosphoryl dolichol might participate as a mannosyl donor in core-PI synthesis but no such evidence was found regarding the possible involvement in the repeating unit assembly. It is suggested that guanosine diphosphate (GDP)-Man donates mannose-1-phosphate (Carver and Turco, 1991; 1992), thereby conserving the α-anomeric configuration of the mannosyl phosphate bond. Thus, the repeating units of LPG appears to be polymerized by the individual alternating transfer of galactose and mannose-1-phosphate residues from their nucleotide derivatives. It has been demonstrated that the assembly of these repeating units of the LPG of promastigote occurs in the golgi body (Bates *et al.*, 1990) and it

has been confirmed using monensin, an inhibitor of golgi function. The addition of other hexoses that comprise side chains of the LPG repeating units of *L. major* and *L. mexicana* has not been examined. One of the key enzyme glycosyltransferases was discovered from *L. major* such as a putative arabinosyltransferase. The arabinosyltransferase may be an important regulatory enzyme in metacyclogenesis in *L. major* (Ng *et al*, 1996; Opat *et al.* 1996).

An LPG deficient strain of *L. major* has been identified which is unable to form the first repeat unit due to a defect in the transfer of the gal residue (McConville and Homans, 1992). This strain express two highly truncated LPG structures on its cell surface (GIPL-4 and GIPL-6) and is unable to survive in mammalian macrophages (Handman *et al.*, 1986).

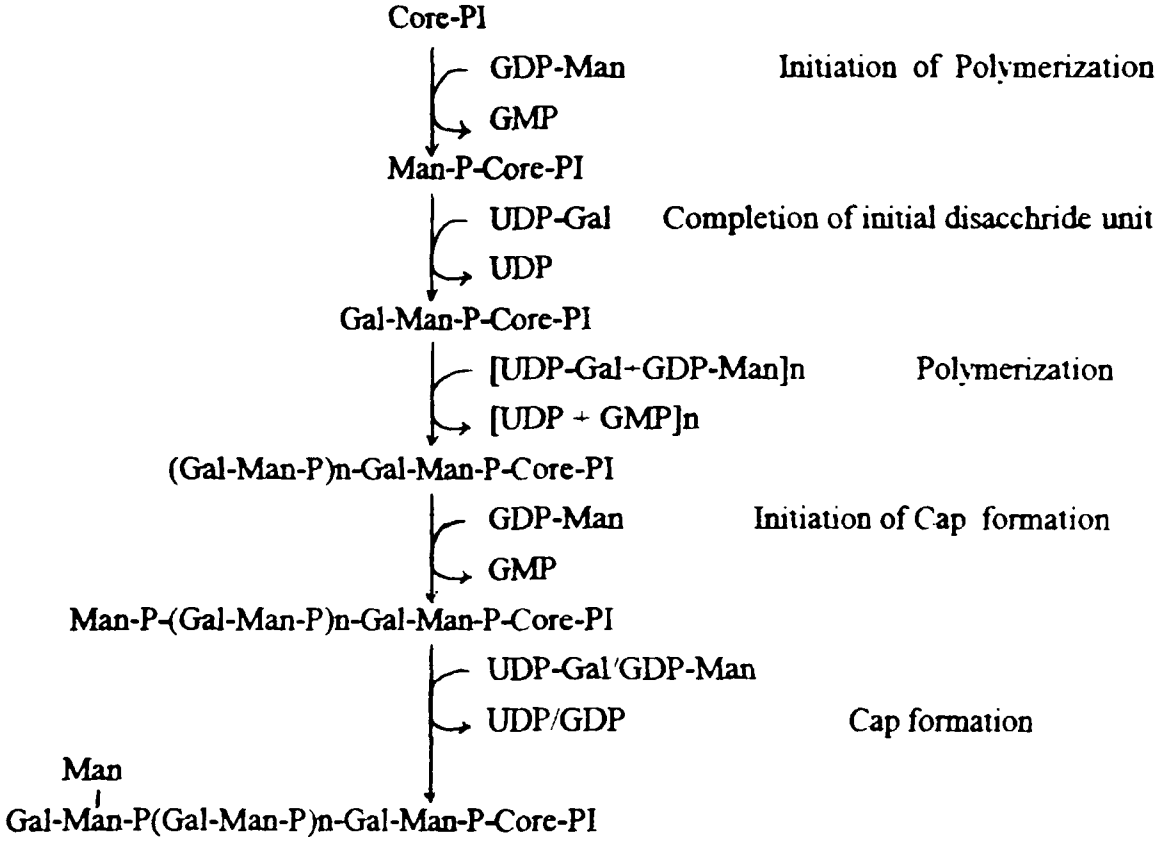


Figure9 is the proposed pathway of assembly of the repeating units and capping oligosaccharides of the *L. donovani* LPG. The core structure is Gal (α 1–>6) Gal (α 1–>3) Galf (β1—>3)-Man-(α 1–>3) Man (α 1–>4) GlcN (α 1–>6) and PI is lyso-1-O-alkyl phosphatidylinositol (Turco & Descosteaux, 1992).

IV. Synthesis of capping Oligosaccharides

Not all but one of the cap structures elucidated from various leishmanial LPGs contain a Man (α 1–>2) Man (α 1) at the reducing end. It is therefore, tempting to speculate on the

existence of a Man (α 1-->2) mannosyl transferase. The activity of this enzyme would result in the signal for cessation of LPG elongation with the formation of a chain terminating Man (α 1,2) Man- α1 containing cap oligosaccharide. As metacyclogenesis is accompanied by an approximate doubling in the size of LPG, a chain terminating mannosyl transferase may prove to be one of the key regulatory enzymes in LPG biosynthesis.

The major GIPL species appear to have a relatively low turnover rate (Proudfoot *et al.*, 1995), in contrast to LPG, which is actively shed from the cell surface and has a high turn over rate (Handman *et al.*, 1984; King *et al.*, 1987). The rate of LPG shedding is increased when parasites are grown in the presence of serum albumin, which contains a hydrophobic pocket (King *et al.*, 1987), and the shed material retains the lysoalkyl PI lipid moiety (Ilg *et al.*, 1992). This is consistent with a non-enzymatic mechanism of release of LPG which pass out of the plasma membrane as either monomers or micelles. This property is probably a reflection of the weak attachment of LPG to the outer leaflet of the plasma membrane via a single aliphatic chain and may be important in allowing the rapid expression of new LPG structures on the cell surface during parasite development (McConville and Ferguson, 1993).

DEFECT IN LPG BIOSYNTHESIS:

LPG-deficient strains of *Leishmania* have provided evidence that LPG is essential for promastigotes infectivity in the mammalian host (Handman *et al.*, 1986; Elhay *et al.*, 1990; McNeely and Turco, 1990). These strains are avirulent in mice and are rapidly killed in the phagolysosome of *in vitro* infected macrophages. It has been demonstrated that intracellular survival of these strains can be prolonged if exogenous LPG is inserted into the promastigote plasma membrane (Handman *et al.*, 1986; McNeely and Turco 1990). In this regard, LPG is thought to prevent complement mediated lysis of promastigote in the blood stream of the host (Puentes *et al.*, 1988), in mediating the initial attachment of promastigotes to the macrophage (Handman and Goding, 1985; Puentes *et al.*, 1988; da Silva *et al.*, 1989, Talamas-Rohana *et al.*, 1990), and also to protect the parasite from hydrolytic enzymes and the oxidative burst in the phagolysosome (El-on *et al.*, 1980; Chen *et al.*, 1989; McNeely and Turco, 1990). The defect of LPG biosynthesis has shown that a mutation occur in the β1-4 galactosyltransferase, which is involved in forming the first repeat unit (McConville and Homans, 1992). Alternative possibilities, involving an impairment in intracellular vesicle transport or a defect in UDP-Gal synthesis or transport are unlikely, as all these Gal containing GIPLs, are still expressed in high copy number at the cell surface (McConville and Bacic, 1989).

The protocol for isolating mutants defecting in the biosynthesis of an abundant cell surface LPG was developed (McConville, 1991; Turco and Descoteaux, 1992; McConville and

Ferguson, 1993; Ilg *et al.*, 1994). LPG is the only major cell surface molecule of Leishmania parasites which terminates in β-linked galactose, and is recognised by lectin ricin agglutinin (King and Turco, 1988). Since mutations in any LPG biosynthetic step, leads to the absence of terminal β-linked galactosyl residues, attached to the parasite, selection against ricin binding should lead to the recovery of a wide spectrum of mutation.

It is observed that R2D2 mutant synthesized a truncated LPG in the glycan core region, and was defective in a step involving addition of galactofuranosyl (Gal_f) there in (Hung and Turco, 1993; Descoteaux *et al.*, 1993; Ryan *et al.*, 1993; Descoteaux et al., 1995; Beverley and Turco, 1995). This type of LPG is called LPG1. The defect in biosynthesis of LPG1 may be functional genetic complementation in *Leishmania* parasites. Thus LPG1 represents a class of gene encoding LPG1 biosynthetic enzymes. It is shown that the LPG made in LPG 1 transfected R2D2 strain was of full length and identical to wild-type LPG in structure (Beverley and Turco, 1995).

The second class of LPG mutants are those which have defective compartmentalization and LPG assembly. They are synthesized as truncated LPG containing only the glycan core and lipid anchor and lacking repeating units (McNeely *et al.*, 1990; Descoteaux *et al.*, 1995). This type of LPG was called LPG2. The accumulation of a truncated LPG core in C3PO indicated a defect in the addition of the first mannose phosphate residue (Descoteaux *et al.*, 1995). However, C3PO microsomal membranes catalysed the addition of repeating unit onto endogenous LPG glycan core acceptors, approaching nearly 65% of wild type levels. The LPG synthesized *in vitro* was of the same size and structure, as that made by wild-type microsomal membranes. These studies indicate that, despite of its LPG- phenotype, C3PO contained all the enzymes necessary to make LPG. Since loss of cellular compartmentalization occurs in preparation of microsomal membranes, it was concluded that LPG2 could affect the localization or compartmentalization of a key LPG biosynthetic precursor or enzyme.

Most probably, LPG2 mediates transport of an essential LPG precursor or biosynthetic enzyme into the secretory network. Interestingly there are several proteins which are tightly associated with LPG, such as the B protein and KMP-11. They are strongly immunogenic protein, which lack typical NH_2- terminal signal peptides (Jardims *et al.*, 1991; 1995; Flinn *et al.*, 1994; Pimenta *et al.*, 1994). Since putatively LPG deficient lines show alteration in the surface localization of B protein (Smith and Rangarajan, 1995), there could be an LPG-coupled pathway for translocation of these proteins into the secretory network involving LPG2. The various roles of LPG2 have implicated regarding the genetic studies, in the *Leishmasnia* infectious cycle. In both *L. donovani* and *L. major*, LPG mutants showed alterations in virulence, as assessed by survival in host macrophages and modulation of the immune response (Handman *et al.*, 1986; McNeely and Turco, 1990; Cappai *et al.*, 1994; Reiner *et al.*, 1994). Thus, the requirement of LPG2 and the

disaccharide phosphate repeats is essential for successful intracellular parasitism of *Leishmania* has been genetically proven (Descoteaux *et al.*, 1995, Beverly and Turco,1995). These studies could provide a useful entry into the intracellular macromolecular sorting and transport in *Leishmania* SPP.

GLYCOSYLPHOSPHATIDYLINOSITOL :

The glycolipids of *Leishmania* parasites belong to the class of glycoinositol phospholipids (GIPL) and they exhibit partial structural homology to the phosphatidylinositol which contains glycolipid membrane anchors of several eukaryotic proteins and the lipid moiety of *L. major* lipophosphoglycan (Rosen *et al.*, 1988; 1989; Elhay *et al.*, 1988). The structural analysis of these glycolipids have indicated close resemblance to the phosphosaccharide core phosphatidylinositol region of LPG of different *Leishmania* spp (see Table 6). Particularly, the *L. major* GIPLs, have a small mannose and galactose containing glycans which are glycosidically linked by an unacetylated glucosamine residues to either 1-O-alkyl-2-acyl-PI or lyso-1-O-alkyl-PI. The glycan parts of these molecules are completely identical to the analogous portions of LPG (McConville *et al.*, 1987; 1990; Schneider *et al.*, 1990; McConville and Bacic,1989; 1990).

The glycoinositol phospholipids (GIPLs) of *L. donovani* promastigotes are non-galactosylated and synthesize GIPLs containing one to four mannose residues in abundance. The *L. donovani* amastigotes GIPLs, contains one to three mannose residues, which are structurally different from promastigote GIPLs and appear to be precursors of the glycolipid anchors of proteins (Fig. 10). The plasma membrane of the amastigotes, also contains a number of glycosphingolipids which are apparently acquired from the mammalian host (McConville and Blackwell, 1991). All newly synthesized GIPL-3 and P-GIPL-3 are rapidly utilized for LPG anchors (Proudfoot *et al.*, 1995). According to author, the supply of GIPL-3 and/ or P-GIPL-3 in *L. major* strain is closely matched by their rate of utilization as LPG/anchors. However, in some *Leishmania* strains, GIPL-3 and/or P-GIPL-3 are found at relatively high abundance on the cell surface (McConville and Bacic, 1989; McConville *et al.*, 1990; 1993). It is possibly due to the regulation of a putative α 1-->6 galactosyltransferase that converts GIPL-2 into GIPL-3.

Cell surface LPG have a relatively rapid turnover, as compared to GIPL. The GIPLs have been observed to be more stable glycoconjugate than LPG (Proudfoot *et al.*, 1995). The high turnover of LPG is due to the rapid shedding of molecules from the cell surface (Handman *et al.*, 1984; King *et al.*, 1987). The shedding of LPG appears to be a stochastic biophysical event, based on monomers of LPG molecules leaving the plasma membrane spontaneously. The shedded LPG molecules have shorter alkyl chains and thus are more polar than the cell associated LPGs (Ilg *et al.*,1992). The stability of the GIPLs containing alkyl-PI lipid moieties is consistent with this

Table 6

Name	Structure	*L major* (P)	*L mexicana* (P)	*L donovani* (P)	*L donovani* (A)
M1	Manα1–4GlcN1–6PI	+	+	–	–
Type-1 GIPL					
M2	Manα1–6 Manα1–4GlcN1–6PI	–	–	–	++
M3	Manα1–2Manα1–6 Manα1–4GlcN1–6PI	–	–	–	++
Type-2 GIPLs					
IM2	Manα1–4GlcNα1–6PI Manα1–3	+	++	++	–
GIPL-1	Manα1–4GlcNα1–6PI Galβ1–3Manα1–3	++	–	–	–
GIPL-2	Manα1–4GlcNα1–6PI Galα1–3Galβ1–3Manα1–3	++	+	–	–
GIPL-3	Manα1–4GlcNα1–6PI Galα1–6Galα1–3Galβ1–3Manα1–3	++	+	–	–
GIPL-A	Manα1–4GlcNα1–6PI Galβ1–3Galα1–3Galβ1–3Manα1–3	++	–	–	–
LPGp	Glcα1—PO_4 6 Manα1–4GlcN1–6*lyso*-PI Galα1–6Galα1–3Galβ1–3Manα1–3	+	++	–	–
GIPL-4*	Manα1–4GlcNα1–6*lyso*-PI Manα1—PO_4—6Galα1–6Galα1–3Galβ1–3Manα1–3	++	–	–	–
GIPL-6*	Glcα1—PO_4 6 Manα1–4GlcNα1–6*lyso*-PI Manα1—PO_4—6Galα1–6Galα1–3Galβ1–3Manα1–3	++	–	–	–
Hybrid-type GIPLs					
iM3	Manα1–6 Manα1–4GlcN1–6PI Manα1–3	–	++	++	–
iM4	Manα1–2Manα1–6 Manα1–4GlcN1–6PI Manα1–3	–	++	++	–
EPiM3	$NH_2CH_2CH_2$—PO_4 Manα1–6 Manα1–4GlcN1–6PI Manα1–3	–	++	–	–

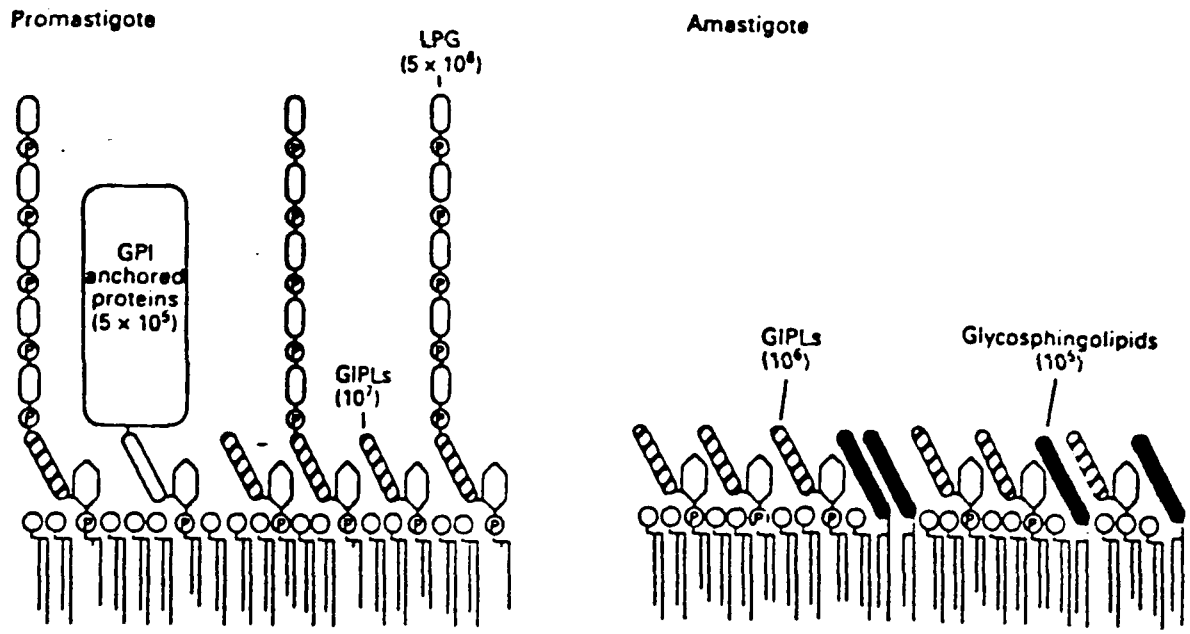


Figure 10: Schematic representations of the cell surface of different developmental stages of *Leishmania* spp.: The cell surface of *Leishmania* parasites is coated by a more complex glycocalyx which also differ in the different developmental stages. The surface glycocalyx of promastigotes stage contains several abundant GPI-anchor proteins, LPG and low molecular-mass GPILs. The surface expression of LPG and the major surface glycoprotein is greatly down regulated in the intracellular amastigote stage (McConville and Ferguson, 1993).

observation as they are much less polar compared to LPGs. Presumably, the Lyso-GIPL species are also sufficiently non-polar to be stable and are associated with the plasma membranes. The difference in turnover of these two classes of surface molecules may reflect their different functions. The high turn over of LPG molecules are required to allow the expression of new LPG structures on the parasite cell surface during their development within the sandfly midgut (McConville *et al.*, 1992). This turnover is thought to be important in regulating the attachment and subsequent detachment of promastigotes from epithelial cells along the midgut (Pimenta *et al.*, 1992; Sacks *et al.*, 1995). In contrast, the slow turnover of the GIPLs is critical in maintaining a protective surface on glycocalyx during and after the differentiation of promastigotes to amastigotes when the levels of LPG and GPI-anchored proteins are drastically diminished (McCoville and Blackwell, 1991; Winter *et al.*, 1994; Bahr *et al.*, 1993; Chneider *et al.*, 1993). GIPL also plays an important role in host-parasite interactions (Proudfoot and Liew, 1995, Jacobson, 1995; Bates, 1995, Aebischer, 1994).

Glycosyl-phosphatidyl inositol (GPI) membrane anchor proteins:

The glycosyl phosphatidylinositol (GPI) anchors are used to anchor proteins to the outer plasma membrane. They can be considered as part of an alternative anchoring mechanism to the transmembrane polypeptide domain of type-1 membrane proteins. Several workers have analyzed the structures and functions of different eukaryotic GPI anchor proteins (Ferguson and Williams, 1988; Low, 1989; Cross, 1990; Thomas *et al.*, 1990, Ferguson, 1991; 1992 b; Ferguson *et al* 1994; McConville, 1991; Turco and Descoteaux, 1992). The GPI anchoring proteins are mostly present at the cell surface of the protozoan parasite, while in mammalian cells, it constitutes only a limited distribution of cell surface proteins. Although some of the plasma membrane proteins of the parasitic protozoa use transmembrane polypeptide anchors (Table 7 and Fig. 11).

All of these surface molecules are placed in GPI family because they contain the structural motif Man α 1-->4 Glc N α 1->6 PI. All GPI-anchor proteins contain common core structure of ethanolamine-PO_4-6 Man α 1-->2 Man α 1-->6Man α 1-->4 GlcN α 1->6 PI. Some of the GIPLs also contain Man α 1->6 Man α 1->4 Glc N α 1->6 PI motif and are called the type-1 GIPLs. Those which diverge from the GPI-anchor core structure by containing a Man α 1->3 Man α 1->4 GlcN α 1->6 PI motif are called type-2, GIPLs. The LPGs structure are also based on a conserved type-2 GIPL core (McConville and Ferguson, 1993). Those which contain the α 1-->6 and α 1-->3 structural feature are called hybrid type GIPLs.

The lipid moieties of the parasite anchors can also vary in a species and stage- specific manner. These anchors contain dimyristoylglycerol (in VSG), lyso-1-O-stearoylglycerol (in PARP) and alkylacylglycerol (in *Leshmania* gp63, and *T. cruzi* IG7 antigen). The *Leishmania* promastigote

Table 7: Occurrence of GPI-anchored proteins in the parasitic protozoa

Species	Protein	Properties
Trypanosoma brucei	Variant surface glycoprotein	Coat glycoprotein of bloodstream trypomastigotes
	Transferrin-binding protein	Surface of bloodstream trypomastigotes
	PARP/procyclin	Coat glycoprotein of procyclic forms
	Procyclic trans-sialidase	Surface of procyclic forms
T. congolense/T. equiperdum	Variant surface glycoprotein	Coat glycoprotein of bloodstream trypomastigotes
Trypanosoma cruzi	Ssp-4	Major surface glycoprotein of amastigotes
	90 kDa 1G7 antigen	Major surface glycoprotein of metacyclics
	gp50-55	Epimastigote/trypomastigote/amastigote antigen
	35/50 kDa/10D8 antigen	Epimastigote/metacyclic antigen; major acceptor of sialic acid
	TCNA/Shed Acute Phase Antigen (SAPA)	120–200 kDa, trans-sialidase/sialidase family (inactive
	GP85 family	Trypomastigote trans-sialidase/sialidase family (inactive)
	F1-160 (160 kDa)	160 kDa flagella antigen
Leishmania spp.	Promastigote surface protease/gp63	Major surface glycoprotein on promastigote surface
	GP46/M2	Promastigote surface
	PSA-2 (promastigote surface antigen-2)	Promastigote surface
Crithidia fasciculata	Protease	Homologue of *Leishmania* gp63
Herpetomonas samuelpessoai	Protease	Homologue of *Leishmania* gp63
Plasmodium falciparum	MSA-1 (merozoite surface antigen 1)	195 kDa, major merozoite surface glycoprotein
	Transferrin binding protein	Merozoite surface
	MSA-2 (merozoite surface antigen-2)	Merozoite surface
	p76 proteinase	Merozoite/schizont surface
Toxoplasma gondii	P22, P23, P30, P35, P43	Major surface proteins on tachyzoite
Giardia lamblia	GP49	Trophozoite surface
Eimeria	Undefined antigens	Surface of sporozoites

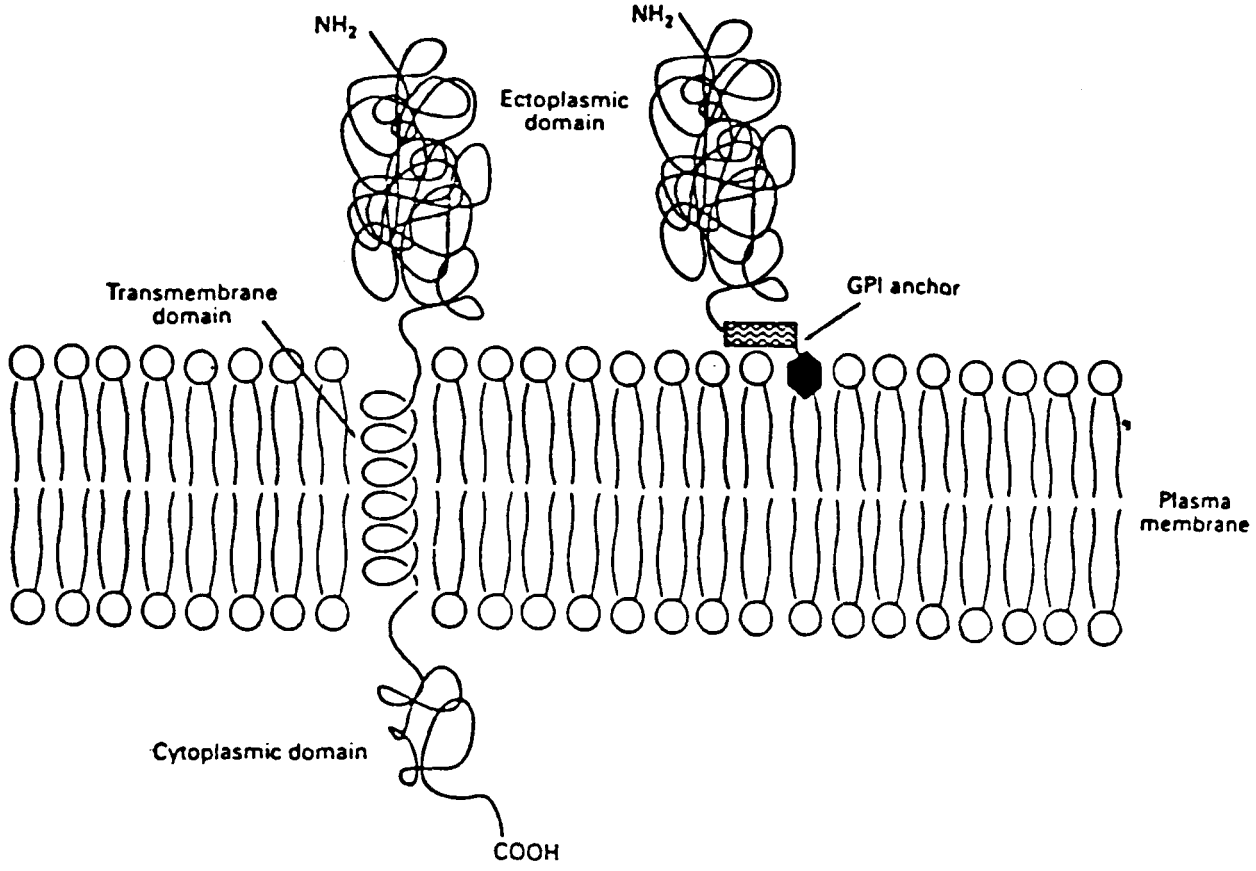


Figure 11: A comparison of transmembrane and GPI-anchored proteins. the type-1 transmembrane spans the phospholipid bilayer while GPI-anchored protein does not (McConville and Ferguson, 1993).

surface contains about 3-5 million copies of LPG (Turco, 1992), which terminate in neutral mannose residues about 20 nm from the cell surface. Together with about half a million GPI-anchored glycoproteins also occur (manly gp63). The LPGs protrude above a dense surface glycocalyx of about 10 million copies of GIPLs (McConville *et al.*, 1993, 1990; McConville and Blackwell, 1991).

The African trypanosome procyclic surface contains neither LPGs nor GIPLs. However, the major surface macromolecule is a glycoprotein called PARP. It is expressed in around 5 million copies/cell. Structurally to African trypanosome has a polyanionic rod domain made up of Glu-Pro repeats, a GPI-anchor and mannose residues in the form of N-linked oligosaccharide about 18 nm from the membrane (Richardson *et al.*, 1989; Clayton and Mowatt, 1989; Roditi *et al.*, 1989). These features are quite similar to the *Leishmania* LPG, in terms of dimension, charge, density and other general physico-chemical properties.

The various function for GPI-protein anchors have been described, particularly in mammalian and protozoan systems and were reviewed by many investigators (Cross, 1990b; Ferguson, 1991; 1992b) (see table 8). It has been suggested that some basic functions are common to higher and lower eukaryotes , while other may represent species specific adaptations that may be advantageous to either unicellular or metazoan organisms.

Surface glycoprotein (GP63):

The major surface protein of *Leishmania* is referred as gp63. It is a glycoprotein of 63,000 Mr containing a GPI membrane anchor (Bordier, 1987; Button and McMaster, 1988; Bouvier *et al.*, 1989; Chaudhuri *et al.*, 1989) and has been reported to be expressed in promastigotes of diverse species of *Leishmania*. It has been demonstrated, to be expressed, in both promastigotes and amastigotes life stages of *L. major* (Frommel *et al.*, 1990) and *L. mexicana* (Medina Acosta *et al.*, 1989); however, all strains may not express an amastigotes form of gp63 (Schneider *et al.*, 1992). It has been demonstrated that gp63 constitute about 1% of the total cellular proteins or half million copies/cell (Bordier, 1987).

GP63 has been shown to be a metalloproteinase (Bouvier *et al.*, 1989; Chaudhuri *et al.*, 1989), with a wide range of pH activity (pH 6-10) and has been reported to hydrolyse non-specifically a variety of substrates including casein, azocasein, gelatin, albumin, hemoglobin and fibrinogen (Chaudhuri *et al.*, 1989; 1990; Bouvier *et al.*, 1989; 1990; Russell and Cross, 1990; Tzinia and Soteriadou, 1991). GP63 shares several characteristics with the membranes of the matrix metalloproteinase family (Nagase Barrett and Woesdsner, 1992) including degradation of components of the extracellular matrix, such as, fibrinogen, location at the cell surface, requirement for Zn^{++} and sequence similarity of the proposed active site, inhibition of the proteinase activity by chelating agents and α_2- macroglubulin (Heumann *et al.*, 1989), secretion as a latent from of the enzyme and the

Table 8: Functions of GPI in mammalian and protozoan cells

FUNCTIONS	MAMMALIAN CELLS	PROTOZOAN CELLS
Attachment of protein to plasma membrane	+	+
Association in membrane micro domains	+	-
Intracellular sorting	+	-
Transmembrane signaling via GPI clusters	+	-
Endocytosis via non-clathrin coated pits(potocytosis)	+	+
High surface expression/low turnover rates	+	+
Selective release of protein by GPI-PLC	+	+
High surface packing	-	+
Contribution of surface glycocalyx	-	+

activation by mercurial compounds (Button *et al.*, 1993).

The genomic organization of gp63 coding genes in the subgenus *Leishmania* have shown various degree of complexity: a tandem array of 6 genes in *L. major* (Button *et al.*, 1989), a cluster of 7 tandem genes with at least 3 dispersed genes in *L. donovani* (Webb, Button and McMaster, 1991), 4 tandemly repeated genes plus 14 genes in *L. Chagasi* (Roberts *et al.*, 1993) and, 2 clusters of 4 and 5 tandem genes respectively together with one separated gene in *L. mexicana* (Medina-Acosta and Russell, 1993a).

The translated amino acid sequence analysis of *L. major* showed that it contains about 602 amino acid in precursor protein consisting of an NH_2- terminal signal peptide of 39 amino acids for membrane targeting, a proregion of 61 amino acids, the mature protein of 477 amino acids containing glycosylation and putative catalytic sites, and a COOH- terminal signal peptide of 20-25 amino acids for GPI attachment (Button and McMaster, 1988). Recent studies have reported that certain gp63 genes exhibit stage-specific expression (Ramamoorthy *et al.*, 1992; Medina-Acosta *et al.*, 1993) such as the *L. mexicana* amastigotes form of gp63 exists as a soluble metalloproteinase in an acidic lysosomal compartment (Ilg, Harbecke and Overath, 1993).

GP63 has also been demonstrated to be a major antigen recognized following infection by *Leishmania* in both experimental animals and humans. Peripheral T cells from a panel of patients with New World Cutaneous leishmaniasis (NWCL) responded to recombinant gp63 (r gp63) and human T-cell lines. Thus, it was conferred that they specifically reacted with rgp63 (Button , Reiner and McMaster, 1991; Russo *et al.*, 1991). Furthermore, gp63 specific T-cells that developed *in vivo* during a naturally acquired infection reacted with gp63 from diverse species of *Leishmania* (Button, Reiner and McMaster, 1991; Russo *et al.*, 1991). The T-cell response to gp63 in cogenic mouse strains were shown to be under the genetic control of specific MHC Class II alleles (Lopez *et al.*, 1991).

FUNCTIONS OF LPG:

The overall structure of LPG and its highly unusual domains indicate that LPG should have several important functions, in the life cycle of the *Leishmania* (Table 9). Evidences have been provided for a surprisingly large number of potential activities that enable the promastigote to survive and flourish in the hydrolytic environments.

EXTRACELLULAR FUNCTION:

Function in Sandfly-*Leishmania* interactions:

LPG plays an important role in sandfly-parasite interactions. During the differentiation

Table 9: Structure/function relationships of *Leishmania* LPG

Function	Probable LPG domain involved	References
Surface coat		
(i) Protection against complement-mediated lysis		Puentes et al., 1990
(ii) Protection against insect/phagolysosomal hydrolases		El-On et al., 1980; Schlein et al., 1990
(iii) Masking of protein antigens		Karp et al., 1991
Cell–cell recognition and attachment		
(i) Binding to receptors in insect midgut	Repeat unit side chains	Pimenta et al., 1992
(ii) Binding to macrophage receptors	Repeat unit side chains	Handman and Goding, 1985; Talamas-Rohana et al., 1990; Kelleher et al., 1992
(iii) Activation of complement and binding to macrophages via the complement receptors CR1 or CR3	Repeat units/mannose cap structures	Puentes et al., 1988; da Silva et al., 1989; Mosser et al., 1992
Survival in mammalian macrophage		
(i) Scavenger of oxygen radicals	Repeat units	Chan et al., 1989
(ii) Chelation of Ca^{2+}	Repeat units	Eilam et al., 1985
(iii) Modulation of macrophage functions		
Inhibition of protein kinase C	Lipid and repeat units	McNeely et al., 1989; Descoteaux et al., 1992
Inhibition of oxidative burst	Lipid and repeat units	McNeely et al., 1990
Inhibition of chemotactic locomotion		Frankenburg et al., 1990
Down-regulation of tumour-necrosis-factor receptors		Descoteaux et al., 1991
Inhibition of IL-1 production		Frankenburg et al., 1990

and multiplication of promastigote form of *Leishmania* parasites that take place in the midgut of sandfly, *Phlebotomus* and *Lutzomyia* spp, the major physical change which occurs during metacyclogenesis is the structural modification of LPG (Sacks *et al.*, 1985). The importance of these modifications with respect to the successful survival of the parasite within the mammalian host has been reported (Sacks, 1989; Turco, 1990). Furthermore, the possibility of these developmentally linked structural changes in controlling the attachment and detachment of maturing promastigotes from midgut epithelial cells has also been speculated (Turco, 1990). The attachment of procyclic promastigotes to epithelium cell lining of its vector is due to the presence of specific receptors on the epithelium cell lining of sandfly. These receptors bind to the LPG molecule of parasites. For example, the multiple β-linked galactose residues of *L. major* LPG is thought to be responsible for the attachment of *P. papatasi*, while other species of sandfly midgut possess receptors of relatively conserved oligosaccharides or 'lectin like' (Pimenta *et al.*, 1994). These lectin like receptors bind to the LPG of procyclic promastigotes. *Leishmania* parasites express two major cell surface molecules, LPG and glycoprotein 63(gp63), during their development. A large amount of procyclic LPG is found on the surface of epithelial cells of the sand fly gut wall. In contrast, despite its abundant expression on promastigotes, gp63 is not detected on the gut cell wall. LPG deficient strain (R2D2, a mutant strain of *L. donovani*) failed to attach to the midgut of the sandfly. The absence of the metacyclic LPG on the gut wall suggest that the modified version of the LPG does not bind to these cells and consequently allows infecting parasite to move forward after their maturation. Hence, LPG is the key determinant which is responsible for attachment and detachment of promastigotes to the sandfly midgut and its migration to the mouth part (Pimenta *et al.*, 1994).

FUNCTION IN BLOOD STREAM :

Complement activation and resistance to complement mediated damages:

During inoculation and infection of macrophages in human blood stream, promastigotes are exposed to the potential lytic effects of normal serum (Joiner, 1988). It appears that the developmentally regulated modifications of LPG represents the major resistance mechanism (Savaira *et al.*,1995; Pimenta *et al.*, 1992). Promastigotes of all *Leishmania* species from log-phase cultures (non-infective) are extremely sensitive to complement mediated lysis. The stationary phase metacyclic promastigotes display an increased resistance to lysis by activation of complement (Franke *et al.*, 1985). It has been suggested that the larger LPG molecules on metacyclic promastigotes are responsible for their resistance to complement mediated lysis. LPG undergoes extensive modifications during metacyclogenesis which includes elongation of the molecule due to an approximate two fold increase in the phosphodisaccharide repeats and reduction in terminal exposed galactose residues. Therefore, elongation of LPG controls the complement mediated lysis. This is supported by the

observation that in *L. major*, most of C5b-9 complexes were spontaneously released from the metacyclic promastigotes surface. This action precludes their insertion into the membrane and death of the parasite (Puentes *et al.*, 1990). The kala-azar serum shows a strong reactivity with a LPG-deficient mutant of *L. donovani* and little reactivity with wild-type promastigotes which confirmed that the humoral response associated with kala-azar does not contribute to immunity (Karp *et al.*, 1991).

Attachment of Host Macrophages:

Since *Leishmania* parasites infect primarily mononuclear phagocytic cells, attachment of parasite to potential host cells require specific recognition molecules on the surface of both parasites and macrophages. The glycoprotein gp63 (Bouvier *et al.*, 1985; 1987) and LPG present on the surface of all the *Leishmania* species, have been implicated as the main parasite ligands (Handman and Goding, 1985, Russell *et al.*, 1986; 1988) whereas CRI, CR3 and Mannose-Fucose receptor (MFR) and $_{p}$150/95 represent the corresponding macrophage receptors (Wilson *et al.*, 1986; 1988; Talamas-Rohana *et al.*, 1990). In *L. major*, the LPG of metacyclic promastigotes is believed to serve as a receptor for the third component of complement (C3), which is then converted to C3b and C3bi by activation of the classical pathway of complement activation and the subsequent CR-1 mediated binding and internalization. The utilization favour of both CR1 and CR3 receptors are implicated to the survival of *Leishmania* promastigotes becuase they promote phagocytosis without triggering the oxidative burst (Wright and Siverstein, 1983).

Studies have further demonstrated that attachment of *L. major* promastigotes to macrophages is inhibited by the Fab fragment of an anti *L. major* LPG antibody, suggesting that LPG is a parasite receptor for macrophages (Handman *et al.*, 1985). The binding of purified *L. major* LPG to macrophage and nonmacrophage cells has been shown to be temperature dependent. Based on these studies two mechanisms of binding have been proposed: a specific mechanism in which the carbohydrate part of the molecule binds to a macrophage receptor, and a non-specific mechanism in which the lipid of LPG interacts with membrane of the cells, probably through insertion into the lipid bilayer. In contrast to *L. major*, *L. donovani* LPG and its delipidated derivative have been shown to bind to a variety to different cell types in a temperature-dependent manner (Tolson *et al.*, 1990).

Talamas-Rohana *et al.* (1990) have shown that the binding of *L. mexicana* LPG to macrophages is mediated by the C-chains of the both CR3 and $_{p}$150/95 receptors which belong to the CD18 family of integrins. The binding site of LPG on CR3 has been observed to be distinct from the binding site of C3bi. These binding studies strongly suggested a role for LPG in the attachment of promastigotes to macrophages (Elhay *et al.*, 1990).

INTRACELLULAR FUNCTIONS:

Intracellular survival in host phagolysosome :

The *Leishmania* parasites have adapted to survive in the highly destructive environment of the phagolysosome, where they encounter different degradative enzymes and other toxic oxygen metabolites. Handman and Greenblatt (1977) provided the first evidence that a parasite excreted factor may be important for the intracellular survival of *Leishmania* parasites. They observed that addition of concentrated excreted factor from *L. enritti* promastigotes cultures promoted the growth of this parasite in mouse peritoneal macrophages, which under normal condition are not permissive.

It is found that LPG is required for intracellular survival of promastigotes. An avirulent clone of *L.major* which lack LPG is phagocytized by macrophages and killed within 18 hours (Handman *et al.*, 1986). Passive transfer of purified LPG from a virulent strain of *L. major* into the avirulent promastigotes confirmed on them the ability to survive in macrophages. LPG deficient variants of *L. donovani* selected for resistance to the ricin agglutinin lectin are phagocytized but can not survive in human monocytes (King and Turco, 1988, McNeely and Turco, 1990). It is therefore suggested that intact LPG molecules is necessary for successful intracellular survival. LPG epitopes can be visualized by immunofluorescence with anti-LPG monoclonal antibodies on the surface of macrophages, as early as, five to ten minutes postinfection and are localized to the immediate area of internalization of the promastigotes (Tolson *et al.*, 1990).

Inhibition of Hydrolytic enzymes :

Adaptation of life in a phagolysosome requires the ability to resist, inactivate or inhibit host hydrolytic enzymes. It has been suggested that survival of *Leishmania* parasites may depend on their ability to inhibit lysosomal enzymes (Alexander *et al.*, 1975) or resistance to lysosomal enzymatic digestion (Change *et al.*, 1976).

Activity of purified LPG against four hydrolytic enzymes from peritoneal macrophages of mice, has been observed. The activity of acid phosphatase, β-glucuronidase and N-acetyl-β-glucosaminidase have not been affected but activity of B-galactosidase is highly inhibited after 3 hr of incubation. The strong negative charge of LPG molecule may account for the observed inhibitory effects. Competitive inhibition by the abundant phosphorylated disaccharide Gal(β1→4) Man in LPG provides an alternative explanation.

The role of LPG in protecting the parasite from digestion by lysosomal enzymes are further shown by measuring the rate of cytolysis of erythrocytes coated and

uncoated with LPG (Eilam *et al.*, 1985). LPG coating significantly diminished the rate of cytolysis by macrophages, suggesting that LPG may indeed enable *Leishmania* parasites to survive in the presence of hydrolytic enzymes.

Inhibitor of Protein Kinase C:

Protein Kinase C (PKC) is a multifunctional protein kinase that specifically phosphorylates serine and threonine residues (Nishizuka, 1986). This enzyme is characterized by a catalytic domain containing an ATP-binding site and a regulatory domain that contains the sites involved incalcium, diacylglycerol and phospholipids binding.

The enzymatic activity of PKC is inhibited by about 50% in presence of a very low concentration of LPG (K_i<1 uM). LPG is the competitive inhibitor with respect to diolein (diacylglycerol) and noncompetative inhibitor with respect to phosphatidylserine (McNeely and Turco,1987). LPG does not affect the catalytic fragment of PKC (called protein kinase M) and cAMP dependent kinase (protein kinase A). The 1-O-alkylglycerol portion of LPG is more effective inhibitor of PKC as compared to carbohydrate portion of LPG, complete LPG molecule, phosphosaccharide-PI, on the basis of molar ratio. The phosphosaccharidyl-PI fragment also hasa greater inhibitory effect than intact LPG and PG (McNeely *et al.*, 1989). The phosphorylated disaccharide fragment (P-Gal β1->4 man α -1) does not affect PKC activity.

In resting cells, a large amount of PKC is cytosolic and inactive. Redistribution and membrane association in general, is a critical step in the regulation of PKC activity. In the presence of elevated intracellular Ca^{++} levels, translocation of the enzyme from the cytosol to the membrane takes place normally in the presence of LPG, indicating that the LPG does not interfere with PKC translocation. In contrast, LPG prevents the phosphorylation of specific PKC substrates, (proteins) in response to DAG. Thus, the inhibitory effect of LPG on PKC may be mediatedby defective protein phosphorylation (Descoteaux and Turco, 1993) (Figure 12). During phagocytosis, PKC is the initiator of the oxidative burst, while LPG inhibits PKC activity in the macrophages. Phosphorlyation and membrane assosiation of the NADPH-oxidase complex components represent the first step of this process (Dewald *et al.*, 1988). The active NADPH oxidase complex catalyzes the one-electron transfer from NADPH to oxygen, generating superoxide anion that is released at the outer surface of the plasma membrane into the extracellular space or into phagocytic vacuoles. Further reduction of the superoxide anions results in formation of hydrogen peroxide, hydroxyl radicals, and singlet oxygen. These products are involved in physiological functions of macrophages, which is elimination of microbes.

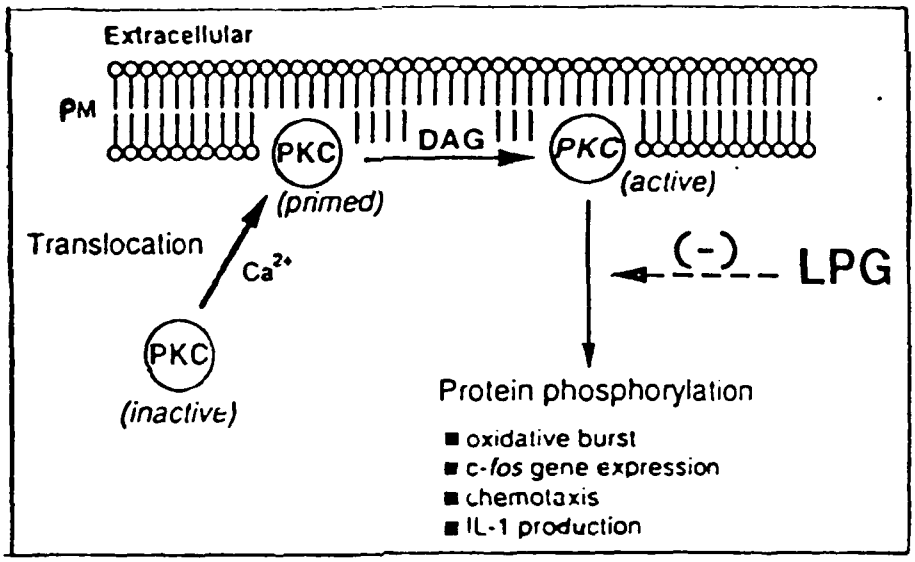


Figure 12: A hypothetical model for the inhibition of PKC -mediated events by LPG (Descoteaux and Turco, 1993).

Products of oxidative burst are deleterious for *Leishmania* promastigotes, in particular H_2O_2 (Murray, 1981; Pearson et al., 1983; Reiner and Kazura, 1982). Therefore, inhibition of PKC activity is important for the parasite to escape from the toxic metabolites of oxidative burst.

Inhibitor of Oxidative burst:

A role of LPG in the impairment of respiratory burst has been su[illegible]. The inhibitory property of LPG on purified PKC may be of major significance, considering the role of PKC in the induction of respiratory burst. Therefore, inhibition of PKC activity leads to the inhibition of oxidative burst. In this regard, phyagocytosis of LPG coated beads inhibits oxygen consumption in monocytes stimulated with phorbol myristateacetate, a synthetic activator of PKC (McNeely *et al.*, 1990; Glew *et al.*, 1988). Using indirect immunofluorescence, LPG or a LPG fragment is detected in the outer membrame of monocytes containing LPG coated beads.Thus, LPG may effectively interact with the monocytes PKC which is involved in induction of oxidative burst.

Scavengers of toxic oxygen Metabolites:

In addition to inhibiting PKC- mediated enzymatic induction of the oxidative burst, LPG may protect promastigotes by scavenging toxic oxygen metabolites generated during the burst, (Chan *et al.*, 1989). LPG is highly effective in scavenging hydroxyl radicals and superoxide anions. The scavenging activity of LPG is largely conferred by the repeating phosphorylated disaccharide units. This property has been explained by oxidation of the hydroxyl groups of sugars to Ketones (Green, 1980).Inhibition of chemiluminescence has also been observed with monocytes treated with LPG but not with the glycosylinositol phospholipid antigens (Frankenburg *et al.*, 1990; El-on *et al.*, 1990), which hasconfirmed the scavenging property of repeating phosphorylated disaccharides. Thus LPG protects the parasite from the damaging effects of oxidative burst through at least two distinct mechanisms: a) attenuation of the PKC- mediated induction of the burst and b) scavenging of the cytotoxic products of the burst.

Inhibitor of c-fos gene expression:

Activation of PKC also results in the expression of several genes, like c-fos gene. The fos-gene function as a nuclear third messenger molecule that regulate gene expression in response to environmental signals. In macrophages,C-fos gene expression is inducible through a PKC or a cAMP-dependent pathway. Activation of PKC results in a rapid and transient increase in c-fos mRNA levels (Radzioch *et al.*, 1987), whereas elevation of c AMP stimulates

a stable and long lasting expression of the c-fos gene (Bravo *et al.*, 1987).

The incubation of macrophages with LPG or its delipidated version resulted in the inhibition of PKC-dependent C-fos gene expression. In contrast, LPG did not inhibit macrophages to express the c-fos gene in response to c-AMP (Descoteaux *et al.*, 1991). This observation is in agreement with the selective inhibitory effect of LPG on purified enzyme (PKC) activity (McNeely and Turco, 1987).

LPG inhibits signal transduction in macrophages:

LPG is involved in several aspects of the parasite macrophage interactions (Turco, 1988). In macrophages, the PKC-dependent signal transduction pathway stimulated by bacterial LPS has been characterized in detail (Hamilton and Adams, 1987). After exposure to LPS, phospholipase C is activated to catalyze the hydrolysis of phosphoinositol 4,5-bisphosphate into inositol triphosphate and diacylglycerol. The latter product activates PKC (Berridge, 1984; Nishizuka, 1984). Activation of PKC results in the phosphorylation of a group of a proteins and in the rapid expression of a set of "early competence" genes, such as the c-fos protooncogene (Hamilton and Adams, 1987). C-fos gene expression is tightly coupled to the activation of PKC, as activators of PKC stimulate a rapid and transient increase in c-fos mRNA levels (Radzioch *et al.*, 1987). Therefore, in macrophages c-fos gene expression represents a stimulus-specific response dependent on the activation of PKC (Radzioch *et al.*, 1987). It has been observed that LPG stimulated the expression of the c-fos gene in a rapid and dose-dependent manner, and to lesser extent it stimulated the TNF gene. This suggest that the initial interaction between LPG and its receptor triggers a cellular response that might influence the subsequent interaction between the parasite and macrophage receptors. Recently, it has been demonstrated that the binding site for *L. mexicana* LPG is conferred, by the α-chains of both CR3 and $P^{150/95}$ (Talamas-Rohana *et al.*, 1990), belonging to the CD18 family of intigrins. From competitive experiments, it is also concluded that *L. mexicana* LPG and *E. coli* LPS share the same binding site on the CD18 family of integrins. Therefore, it is also possible that the cellular response to *L. donovani* LPG involve CR3 and/or $P^{150/95}$. Pretreatment with *L. donovani* LPG is capable of inhibiting in a dose dependent manner subsequent LPS-induced c-fos gene expression, but not the LPS-induced TNF gene expression (Descoteaux *et al.*, 1991). They demonstrated that pretreatment with LPG interfered with the signal trnasduction pathway leading to the expression of the c-fos gene in a selective rather than a general manner. The LPG is a competitive inhibitor with respect to diolein (McNeely and Turco, 1987) which suggest that LPG, or a fragment of this molecule, interferes with the physiological process of PKC activation upon generation of diacylglycerol. Hence, LPG of *Leishmania* parasite appear to inhibit the PKC-dependent

signaling pathway, which plays a major role in the activation process of macrophages (Fan *et al.*, 1988; Schultz *et al.*, 1990; Descoteaux *et al.*, 1991).

Inhibitor of Chemotaxis:

L. donovani LPG and its delipidated counterpart are potent inhibitors of monocyte and neutrophil chemotactic locomotion. Inhibition of inflammatory reactions in the lesion sites may contribute to the chronicity of the disease. (Frankenburg *et al.*, 1990). Chemotaxis activity of neutrophil or monocyte is inhibited due to alteration of PKC activity.

Inhibitor of Viral Fusion:

LPG of *L. donovani* is a potent inhibitor of viral fusion. The LPG used for viral fusion is introduced into human erythrocyte ghost (HEG) membrane. When LPG is incorporated in a very low concentrations into intact human erythrocyte membrane, it strongly inhibited sendai virus and induced hemolysis. LPG incorporated in HEGs, reduced the binding of both sendai and influenza viruses to HEG. There fore, it strongly inhibited over all viral fusion (Ling *et al.*, 1995). It has been suggested that LPG stabilized the bilayer structure of phosphatidylethanolamine against the formation of an inverted-hexagonal structure. LPG may give rise to an effective 'steric repulsion'between the viral and HEG membranes, thereby modulating some specific modes of interaction between viral target membranes in the overall fusion process. LPG may also modulate the binding rigidity of the HEG membrane in the direction of making the destabilization and rearrangement of the underlying lipid bilayer (Ling *et al.*, 1995).

Inhibitor of IL-I Production:

LPG has been implicated as an inhibitor of interleukin-1 (1L-1) production. Two macrophage accessory functions regulate T-helper lymphocyte activation: a) the expression of major histocompatibility complex class II molecules and b) the production of interleukin (IL -1) (Unanue *et al.*, 1987). In *L. donovani* infected macrophages, these two functions are defective (Olivier and Tanner, 1989; Reiner, 1987; Reiner *et al.*, 1987). Incubation of monocytes with purified *L. donovani* LPG inhibited lipopolysaccharide (LPS) induced IL-1 secretion (Frankenburg *et al.*, 1990).

Chelator of Calcium :

Calcium plays an important role in the regulation of cellular functions, mainly as an intracellular second messenger and as an enzyme cofactor. It has been shown that *L. major* infected macrophages contain approximately 40 % more exchangeable calcium than uninfected controls, as well as macrophages engulfing LPG-coated erythrocytes have increased levels of calcium compared to macrophages engulfing control erythroytes (Eilam *et al.*, 1985).

In recent studies using NMR the effect of calcium on the tertiary structure of the glycan moiety of LPG was examined. The investigators concluded that calcium does not perturb the three dimensional structure of the glycan and it binds to LPG in the vicinity of the phosphate groups. Therefore, the ability of LPG to chelate calcium may have important implications with respects to the ability of *Leishmania* parasites to survive within macrophages (Homans *et al.*, 1992).

Modulator of TNF α Receptors:

LPG also modulates TNFα receptors. TNFα activates macrophage cytotoxic functions and the subsequent destruction of the invader. It also plays a role in reducing the ability of macrophages to bind TNF α. Although, the *Leishmania* parasites stimulates macrophages to secrete TNF α (Green *et al.*, 1990), a down regulation of TNF α receptors may play a role in the survival of *Leishmania*, particularly during the initiation of infection (Descoteaux *et al.*, 1991; Green *et al.*, 1990).

LPG as a Candidate Vaccine:

LPG is the most abundant surface molecule of *Leishmania* promastigotes cell membrane. Purified *L. major* LPG when administered to genetically resistance mice, in which cutaneous lesions resolve spontaneously, induced full protection against a challenge with promastigotes, whereas partial protection was achieved in the susceptible mice (Handman and Mitchell, 1985). This observation suggested that LPG might be considered as a condidate vaccine antigen against cutaneous leishmaniasis.

Incorporation of LPG into liposomes proved to be an efficient way to immunize mice against *L. mexicana* without causing any exacerbation of the disease (Russell *et al.*, 1988). Adaptive transfer of T-cells isolated from immunized mice into syngenic mice provided protection against a challenge with *L. mexicana* promastigotes, indicating that protection is a function of antigen specific T-cells.

Several researchers have demonstrated that T-lymphocytes play a dominant role in the acquired resistance to *Leishmania* parasites. Mice vaccinated with *L. major* LPG contained an increased frequency of *L. major* reactive T-cells (Moll *et al.*, 1989). Furthermore, LPG induced a specific delayed type hypersensitivity in *L. major* infected mice, which also produced T-cell dependent IgG to LPG. It has been suggested that T-cell can recognize and respond to LPG, although it did not respond *in vitro*.

Mendonca *et al.*, (1991) have reported that T-lymphocytes from cutaneous leishmaniasis patients responded to highly purified *L. brazilensis* LPG, whereas proteinase K treated LPG did not stimulate any response. This important observation indicated that stimulation of T-cell responses by LPG may infact be induced by tightly associated protein contaminants. Purification and partial characterization of the LPG associated protein contaminants revealed the presence of several proteins. It has been demonstrated that peptides are potent stimulators of T-cells *in vitro* as well as, from mice immunized with protein contaminated LPG for leishmaniasis (Jardim *et al.*, 1991; Russo *et al.*, 1992). Whereas protein free LPG failed to stimulate any T-cell responses. Thus, LPG does not appear to be able to elicit T-cell response, it may act as a natural adjuvant for the proteins with which it is tightly complexed.

LPG used for Serotyping:

Several polyclonal and monoclonal antibodies generated against *Leishmania* strains react with epitope present on LPG (De barra *et al.*, 1982; Greenblatt *et al.*, 1983). These antibodies have proved useful for serotyping *Leishmania* strains which selectively precipitate the excreted form of LPG. However, monoclonal antibodies that are initially

believed to recognize the core region of *L. donovani* LPG are subsequently found to react with protein contaminants (Jardim *et al.*, 1991; Tolson *et al.*, 1989). It has been shown that an anti-LPG mAb (CA7AE) binds specificially to the repeating phosphorylated disaccharide epitope of the LPG molecule. The repeating phosphorylated disaccharide epitope of *L. donovani* LPG appears on the surface of infected macrophages as early as 5 to 10 min post infection. Thus, LPG epitopes are detected on the amastigotes and the infected macrophages by a number of monoclonal antibodies. Although, these infected macrophages expressed LPG epitope which is distinct than parasite LPG expressed on promastigotes and amastigotes (Handman, 1990; Tolson *et al.*, 1990). The kala-azar patient serum contained antibodies which are specific for surface components, especially LPG. These antibodies bind to live promastigotes in absence of LPG expression which suggest that LPG is a mask for the surface antigen (Karp *et al.*, 1991). The antibodies against LPG are detected in 45 kala-azar serum out of 50 samples (Krutzhals *et al.*, 1992). Antibodies are purified from kala-azar serum by affinity chromatography. These antibodies strongly reacted with LPG of *L. major* amastigotes and to a lesser extent with the LPG of promastigotes (Osborn *et al.*, 1994). This type of reactivity could be directed against β1-->3 galactosyl determinants shared common epitope for promastigotes and amastigotes. Hence, LPG is used for serotyping of leishmania from serum.

CHAPTER 3

PARASITE MAINTAINANCE AND INFECTIVITY

INTRODUCTION:

The species of genus *Leishmania* are a biologically diverse group of trypanosomatid flagellates. All of them are transmitted by *Phlebotomine* sandflies. They live extracellularly in the insect's digestive tract, where they undergo a morphological change to form promastigotes. When inoculated into the mammalian host by the vector, they infect macrophages, and then differentiate into nonmotile amastigotes that multiply intracellularly (Chang, 1983). Regular availability of parasite is a prerequisite for any experiment. The promastigote stage has been widely cultured *in vitro* in a variety of biphasic and monophasic media (Chatterjee, 1957; Trotter *et al*; 1980a; 1980b; Bhatnagar *et al*; 1989) and the amastigote stage, by regular passage in susceptible animal host (hamster or BALB/c mice). The long term *in vitro* cultivation of promastigote decreases the virulence of *Leishmania* (Giannini, 1974; Neal, 1984). The loss of infectivity of very old cultures may be due to the loss of the potential to form metacyclic promastigotes (da Silva and Sacks, 1987). For maintaining infectivity during the course of *in vitro* culture, it is necessary to passage parasites in susceptible animal host. Recent culture techniques for *Leishmania* promastigotes offer a new and more confident opportunity for diagnostics, biochemical, metabolic, immunological and chemotherapeutical investigations.

The parasite is exposed to growth conditions that differ significantly within vector and the host with respect to temperature, pH, nutrients and serum components. The parasite survival is facilitated by mechanism such as pH and temperature tolerance morphogenesis, resistance to complement lysis and changes in surface molecule. Cells from higher eukaryotic organism can not tolerate drastic changes in pH and temperature. Despite the induction of stress responses such responses are transient and cannot always prevent cell death. In contrast, *Leishmania* parasites encounter and survive extreme environmental conditions as an integral part of their life cycle. Extreme variations both in pH and temperature can trigger changes in gene expression in *Leishmania* that result in the development of new life forms (Zilberstein *et al.*, 1991; Shapira *et al.*, 1988; Bates, 1992; Leon *et al.*, 1995).

Leishmania parasites are highly adaptable to the different environmental pH. As determined by various enzymatic assays promastigotes of *L. donovani* metabolize glucose, proline and nucleotides most rapidly at pH 7.0-7.5. On other hand, amastigotes catabolize these substrates at an optimal pH of 4.5-5.0 (Mukkada *et al.*, 1985).

Although the pH of the extracellular and intracellular environments of *Leishmania* parasite differ by upto 2 pH units, the intra cellular pH of the parasites remains constant throughout their life cycle. The observations that promastigotes and amastigotes function best at their corresponding environmental pH values suggests, that they have developed mechanisms to sense changes in environmental pH (Zilberstein, 1991; Zilberstein and Gebstein, 1993).

It has been suggested that the adaptation of survival of *Leishmania* parasites in various pH unit during the growth is phenotypic rather than genotypic (Zilberstein and Shapiro, 1994). pH of the growth medium significantly influences various important function of parasite like, induced gene expression or DNA and protein synthesis, low pH induced metacyclogenesis, transport of various ions. Recent studies demonstrated that the ability of *L. donovani* promastigotes to regulate their intracellular pH depends on the presence of chloride ions in their cytosol (Vieira *et al.*, 1994). Glaser *et al.* (1988) found, using phosphate NMR and 5,5-dimethyl-2-4-oxazolidinedione (DMO), that promastigotes and amastigotes maintain similar intracellular pH values of 6.8-7.4 throughout an extracellular pH range of 4.5-7.5 (Zilberstein and Shapiro, 1994).

Exposing promastigotes of species that transform morphologically into amastigotes like cells at 35°C causes a reduction in their metabolism (Biegel *et al.*, 1983; Hunter *et al.*, 1984). This reduction is probably due to reduce, cell proliferation although the cells remain viable for atleast 72 hrs. Proliferation of promastigotes that transform morphologically *in vitro* occurs only at temperature less than 33-34°C (Bates, 1993). Although amastigotes are usually obligatory intracellular orgonisms and can be cultivated under axenic conditions using some combinations of temperature, pH, and serum concentration (Bates, 1992; 1993; 1994; Pan, 1984; Doyle *et al.*, 1991; Eperon and McMahon-Pratt, 1989). The exposure of cultured *Leishmania* promastigotes to temperatures typical of human hosts leads to an accumulation of hsp 70 and hsp 83 transcripts (Shapira *et al.*, 1988; Vander Ploeg, 1985). The combined effects of temperature elevation on parasite morphology, protein synthesis, and pattern of specific transcripts suggests that temperature is a factor for differentiation in *Leishmania* (Zilberstein and Shapira, 1994).

The past few years have witnessed a surge in interest in lectin-parasite interactions. The lectins have become valuable tools to study the insertion, fate, distribution and functions of glycoconjugates on and in parasites. The lectins are carbohydrate binding proteins or glycoproteins other than enzymes or antibodies (Goldstein and Hayes, 1978) and have been used in the identification of exposed surface glycoconjugates because of their ability to noncovalently and relatively specifically binds to saccharide residues (Jacobson and Doyle, 1996). All parasites have carbohydrates on their surfaces as part of their cytoskeletons or in their internal structures. These cell surface molecules bind with lectins and agglutinate the organisms. The agglutination, defined as decreased number of free promastigotes or increased clumping of promastigotes to each other as compared to control, occurs with lectins like, Con-A, RCA, SBA and PNA.

The agglutination of *Leishmania* by carbohydrate-binding lectins has been extensively studied. Lectin binding studies of *Leishmania* species (Dwyer, 1974; 1977; Doran and Herman, 1981; Hernandez, 1982; Schottelius, 1982; Gueugnot *et al.*, 1984) have found in

general that promastigotes of *L. donovani, L. major, L. mexicana, L. tropica, L. braziliensis* and *L. aethiopica* are agglutinated by Con A and RCA. An increase in promastigotes infectivity has also been observed with developmental change from logarithmic phase to stationary phase during growth in axenic cultures (Giannini 1974; Franke *et al.*, 1985; Sacks *et al.*, 1985). The development of such infective or metacyclic promastigotes in axenic cultures is associated with changes in lectin binding cell surface molecules (Sacks *et al.*, 1985), altered mobility and increased resistance to killing by normal human serum (Franke *et al.*, 1985; Sacks *et al.*, 1985). These changes demonstrated by the work of Sacks *et al* (1985) with culture of *L. major* but comparable differences in lectin binding were not observed in *L. donovani*. The log phase promastigotes of *L. major* were 100% agglutinated, whereas stationary phase (metacyclic form) or infective promastigotes failed to bind PNA due to some alterations in surface carbohydrates accompany the development of promastigotes into an infective stage. It has also been demonstrated that lectins reacted with a broad band of membrane-associated components (Jaffe and McMahon-Pratt, 1988), which is similar to that observed for a phosphorylated galactosyl-β- mannose polysaccharide from *L. donovani* (Turco *et al.*, 1984); for isolated excreted factor from *L. major* (Handman *et al.*, 1984), a polysacchride containing glucose, galactose, mannose, phosphate and sulphate (Palatnik *et al.*, 1985) and for a lipopeptidophosphomannan containing mannose and galactose isolated from *L. adleri*.

MATERIALS AND METHODS:

ANIMALS:

Healthy rabbits (CDRI strain) 3-4 months old, weighing 2.5-3.0 kg, were used for blood collection for the preparation of bi-phasic culture media. BALB/c mice, weighing 20-22 g, reared and in bred at the animal house facility at CDRI, Lucknow, were used for isolation of peritoneal macrophages. Male syrian golden hamsters (Mesocricetus auratus), 35-45 gm in weight,were used for *in vivo* studies.

PARASITE STRAINS:

Leishmania donovani strain UR6 (MHOH/IN/1978/UR6) was isolated from kala-azar patients in India and obtained from Prof. A.N.Bhaduri of Indian Institute of Chemical Biology, Calcutta, India. It was cultured on Brain Heart Infusion Agar with rabbit blood preferably in semi-solid and biphasic media, as well as in liquid monophasic medium (L_{15}).

Parasite *L. donovani* (strain MHOM/IN/80/Dd8), originally isolated from Bihar, India, in 1979, was subsequently obtained through kind courtesy of Prof. P.C.C. Garnham of Imperial College, London in 1981. This strain is being regularly maintained *in vitro* in

monophasic and biphasic media and in *in vivo*, in golden hamster (Mesocricetus auratus).

Maintainence of parasites:

Parasites: The *Leishmania donovani* promastigotes, (strain UR6) presently nonpathogenic, (Ghosh *et al.*, 1985; Mukherjee *et al.*, 1988) was cultured at 22° ± 1°C on brain heart infusion agar supplemented with rabbit blood as solid media (Chakraborty *et al.*, 1988; Mukherjee *et al.*, 1988) and maintained by subculturing at 72 hr intervals. (Chakraborty *et al.*, 1988)

The *L. donovani* promastigotes of Dd8 strain (MHOM/IN/80 Dd8) was grown at 26±1°C in monophasic and biphasic media (Chatterjee, 1957; Bhatnagar *et al.*, 1989; Mandwal *et al.*, 1988; Anuradha *et al.*, 1992; Gupta *et al.*, 1992) and maintained routinely in this laboratory. After every few subcultures, the strain is passaged through hamsters *in vivo* to retain its infectivity/viability which is quite often diminished by continuous *in vitro* culture.

PREPARATION OF MEDIA:

Semi-solid medium:

The composition of solid medium was:

Brain heart infusion powder	24.0 g
Agar powder	9.0 g
TDW	600.0 ml
D-glucose	10.8 g

Preparation of medium in brief, is as follows: Brain heart infusion and agar powder were dissolved into 500 ml TDW and autoclaved at 10 lbs pressure for 10 min. The medium was allowed to cool upto 50-55°C, pH was adjusted to 7.4±0.2 with NaOH. D-Glucose was dissolved in 100 ml of TDW. The medium and D-glucose solution were sterilized at 15 lbs pressure (121.6°) for 15 min. Fresh rabbit blood (5-6 ml) was taken under sterilized conditions and added to warm D-glucose solution. To this was added 50 I.U./ml of benzyl penicillin (30,000 I.U.) and 50 µg/ml (30 mg) of streptomycin. The glucose solution was then added to the medium, mixed thoroughly and poured into sterilized petri dishes or culture tubes. These were then kept at room temperature for 2 days. The sterility was checked after 24 hr at 37° and finally stored at 4° C till use (Saha *et al.*, 1986).

Liquid medium:

The composition of liquid media is:

Medium 199 or Medium RPMI-1640	11.0 g

HEPES buffer (25mM)	5.95 g
$NaHCO_3$	2.25 g
TDW	900 ml
D-glucose	2.0 g
Gentamycin	40 mg
Fetal Calf Serum (FCS)	100 ml

All the above ingredients, were dissolved in 900 ml of water and stirred for 1hr on a magnetic stirrer at room temperature. The pH of the medium was adjusted to 7.2 ±0.2 with the help of 1N HCl or 1N NaOH. The medium was then filtered through sartorious millipore filter (0.2 µm pore size) and kept at 37° for 24 hours for sterility checking. The medium was finally supplemented with 10% FCS and gentamycin (40 µg/ml) and stored at 4°C till use.

Leibovitz (L_{15}) medium:

The compositiom of L_{15} medium

Leibovitz medium powder	14.8 g
D-glucose	2.0 g
Tryptose phosphate broth	2.95 g
TDW	900 ml
Serum (FCS)	100 ml
Gentamycin	40 mg

The Leibovitz medium (L_{15}) (Sigma) and 2.0 g D-glucose were dissolved in 800 ml of TDW to which 100 ml of tryptose phosphate broth was added as an extra supplement. Tryptose phosphate broth was prepared separately, dissolving 2.95 g of tryptose phosphate broth in 100 ml TDW and autoclaved at 15 lbs for 15 mins. The pH of the medium was adjusted to 7.2±0.2 and filtered through sartorious millipore filter (0.2 µm pore size) and kept at 37°C for 24 hours for sterility checking. The filtered L_{15} medium was finally supplemented with autoclaved TPB(Hi-media), 100 ml of FCS and gentamycin. It was stored at 4°C, until use.

Biphasic media (NNN Medium):

This medium consists of four parts of salt bactoagar and one part of defibrinated rabbit blood.

Preparation of N.N.N. medium:

Bacto - Agar (Difco)	15.5g
Sodium chloride	6.6g
Triple distilled water	1000 ml

Bacto-Agar and sodium chloride were mixed thoroughly by warming at 60-70°C and the pH adjusted to 7.2. The mixture was finally autoclaved at 15 lbs pressure for 15 min. Antibiotic, i.e. gentamycin was added to a concentration of 40 µg/ml and stored at 4°C till use.

Defibrinated rabbit blood was mixed in a ratio of 4:1 (4 ml of molten agar: 1 ml of defibrinated rabbit blood) in screw capped vials at temperature not more than 60°C. The bactoagar and defibrinated rabbit blood was mixed thoroughly by gently rolling the vials between the palms (Chatterjee, 1957).

The vials were kept at 37°C for 24hr to generate water of condensation. These NNN medium vials were finally stored at 4°C until use.

Overlay of Medium:

RPMI-1640 medium:

RPMI- 1640 powder	10.4 g
HEPES buffer	5.94 g
$NaHCO_3$	2.25 g
D-Glucose	2.00 g
TDW	900 ml

Powdered RPMI-1640 medium (Sigma) with glutamine but without bicarbonate ion was used. All the above ingredients were dissolved in 900 ml of TDW. The solution was stirred for 1 hr on a magnetic stirrer. The pH of the medium was adjusted 7.2±0.2 and sterilized by filtering through a millipore filter of sartorious (0.2 µm pore size). Gentamycin was added at a concentration of 40 µg/ml after sterility testing and stored of 4°C till need.

Lock's solution:

Sodium chloride	8.0 g
Potassium chloride	0.2 g
Calcium chloride	0.2 g
Potassium dihydrogen Phosphate	0.3 g
D-Glucose	2.5 g
TDW	1000 ml

All the above ingredients were dissolved into 1000 ml of water and stirred on magnetic stirrer for an hour. The pH of the solution was adjusted 7.2±0.2 with the help of 1N HCl or 1N NaOH. The solution was sterilized by passing through 0.2 µm sartorious membrane filter. 2.0 ml of stock solution was poured in thioglycolate medium (3 g in 100 ml) and kept at 37°C for 24 hr for sterility test. Finally to it was added 1.0 ml of gentamycin (40 µg/ml) and stored at 4°C until use.

***In vitro* maintenance of the parasites:**

Promastigotes of both the strains UR6 and Dd8 were grown *in vitro* in various media.

Semi-Solid media:

To the culture tube containing approximately 5 ml of solid media, 6-8 colonies of *L. donovani* promastigotes (UR6) were inoculated and 10-16 colonies were inoculated on the petri dishes for solid media. These colonies were gently spread on the slant by culture loop. The culture tubes and petri dishes were incubated at 22±1°C in a BOD incubator for 72 hr or 3 days. The viability of the organisms were routinely checked by taking few parasites from culture tubes or petri dishes on the glass slide in normal saline or phosphate buffer and observing under the microscope (Saha *et al.*, 1986)

Liquid media:

Leishmania donovani Promastigotes (Dd8) were maintained in (monophasic)liquid media. 0.2 ml of $1.0x10^7$ cells/ml were inoculated into the culture vials or tubes containing 4-5 ml of liquid RPMI-1640 or L_{15} media. Thus each vial contained $0.5x10^6$ promastigotes/ml. Tubes were incubated at 26±1°C for 4-5 days in a BOD incubator. The subcuturing was carried out after 5 day in L_{15} and 7 days in RPMI-1640 medium. Monophasic media are primarily used for mass cultivation of promastigotes.

Biphasic media:

Prior to subculture, RPMI-1640 and NNN medium (Bactoagar base) tubes were brought to room temperature and 3-4 ml of overlay (RPMI-1640) was added in each vial or tubes. Positive axenic cultures containing $1-2x10^6$ promastigotes of stationary phase (matacyclic form) were inoculated in each vial aseptically. These culture vials were incubated at 26±1°C for 4-5 days. These parasite passes through four different growth phases during the course of cultivation viz. (i) lag phase (ii)logarithmic growth phase (iii) stationary phase (iv)decline phase (Chang and Hendricks, 1985). Subcultures of the parasites were carried out every week for continuous maintenance.

***In vivo* maintenance of Parasites:**

The parasites were maintained *in vivo* in hamsters through amastigotes to amastigotes by syringe passage or at times by inoculating the stationary phase promastigotes.

Preparation of infective inoculum :

Heavily infected hamsters (50-60 days post infection) were sacrificed, their spleen

removed aseptically in RPMI- 1640 medium and cut into small pieces. These were homogenised lightly in sterile RPMI-1640 medium with the help of motor driven tissue homogenizer, consisting of a glass tube and a teflon pestle. The suspension was initially centrifuged at 500Xg for 10 min at 4°C to settle all tissue debris. The supernatant was collected and recentrifuged at 1500Xg for 30 min at 4°C. After this the supernatant was discarded and the sediment containing amastigotes was resuspended in RPMI-1640 medium. The number of amastigotes were counted in Neubaur's haemocytometer. The inoculum size was adjusted so as to contain $1x10^7$ parasites in 0.1 ml of suspension.

Inoculation of animals :

Male hamsters weighing 35-45 g were inoculated with $1x10^7$ amastigotes intracardially (Stauber *et al.*, 1958). The infection gets established in the spleen in about 20-25 days post inoculation. But when $1x10^7$ promastigotes was used for inoculation the infection in the spleen of hamsters occured in about 45-60 days post inoculation.

Growth curve :

For ascertaining the growth of *L. donovani* promastigotes in culture, the growth studies of promastigotes of different strain were carried out. Four strains of *L. donovani* Dd8 (MHOM/IN/80/Dd8), Ag83 (MHOM/IN/83/Ag83), RMRI (MHOM/IN/90/RMRI168) and SS (isolated from Patna, India, and received from RMRIMS, Patna, India) were used in this studied.

1.9 ml of complete medium was put into a sterile screw capped 15 ml vial (Borocil India). It was inoculated with 0.1 ml of inoculum, in which the parasite count was adjusted to 10^6 parasites/0.1 ml. Hence, each vial contained $0.5x10^6$ parasites/ml. The same ratio of medium to parasite was maintained for mass cultivation of parasites.

Harvesting and storage of parasites:

Leishmania donovani promastigotes (strain UR6) cells were removed from the petri dishes as well as from culture tube using culture loop after 3-4 days of inoculation and suspended into PBS(0.1M, pH 7.4) or normal saline. *Leishmania donovani* promastigotes strains Dd8, Ag83, RMRI168 and SS were cultured in L_{15} monophasic medium with 10% FCS in presence of gentamycin (40 µg/ml). These cells were harvested after 4-5 days of inoculation. The growth of the parasite was checked under microscope. The culture media containing the parasites or parasites suspended in PBS (0.1M, pH 7.4) were centrifuged at 1500Xg for 10 min at 4°C. The supernatant was removed and the pelleted cells were collected

and washed three times with PBS (0.1M,pH 7.4) or normal saline. These parasites were used for experiment immediately or stored -20°C according to requirement of the experiments.

The slide smear was prepared by overlay of the parasite on albumin coated slide and dried. The smear was fixed in methanol for 5-10 min. The staining solution (10% Giemsa stain) was layered on the slide and left for 30 min. The slide was then washed with running water followed by TDW. The stained smear was examined under oil immersion with a microscope.

Agglutination studies:

Stock solution (1.0 mg/ml) of lectins PNA, PHA-P. Con-A and RCA_{120} were prepared in TDW.

RCA_{120} lectin was prepared in 0.005 M sodium phosphate and 0.2 M sodium chloride pH 7.2, containing 0.1% sodium azide. These solutions were diluted to 1 µg/ml of final conc. by serial dilution method with PBS (5 mM phosphate, 0.2 M NaCl, pH 7.2).

50 µl of parasites suspension ($1x10^7$ cells/ml) was added to 50 µl of lectin solutions and mixed thoroughly and then incubated at 25°C for 30 min. Controls were prepared in the absence of lectins solution or in the presence of specific inhibitor of lectins. Agglutination was observed for different strains at various concentrations of lectins by counting on haemocytometer under microscope. Permanent slides were prepared as smear on the slides and fixed in methanol and dried. The slides were stained with 10% Giemsa stain for 30 min., washed, dried and examined under light microscope in presence of oil immersion and photographed.

RESULTS AND DISCUSSION:

To culture promastigotes *in vitro* conditions it is necessary to start the primary culture. by sacrificing a heavily infected animal and removing its spleen, under asceptic conditions. The primary culture was started from the infected spleen pieces into biphasic media at 26 ± 1°C. Approximately 5×10^5 promastigotes per ml were obtained from each culture tube in log phase which were added to fresh culture medium. If slight haziness was observed in any tube it was discarded because it indicated the presence of contamination. From time to time, small amount were taken asceptically and examined under higher power of a light microscope for accessing their mortality. The parasites multiply by longitudinal binary fission producing a large number of flagellates which were subcultured weekly.

***In vitro* culture and maintenance of promastigotes:**

In vitro culture and maintenance of *L. donovani* promastigotes were carried out in the following media.

1. **Semi-solid media**: *Leishmania donovani* promastigotes (strain UR6) were cultured on the brain heart infusion agar supplemented with rabbit blood in semi-solid as well as liquid media (Chakravorty *et al.*, 1988; Saha *et al.*, 1986). **Plate 1** shows the growth of the parasite in semi-solid medium. These parasites developed as colony like structures on the petridish plate. The shape and size of colony of *L. donovani* were undefined. They were oval, round and other shaped containing dimensions 2 x 4 mm. This strains of *L. donovani* had a property of growing in clustors. All surface area of the petri dish containing parasites looked like a colony or cluster on the petridish plate due to this specific property of strain. The purity of the culture and the growth of parasites were checked after 72 hrs. The yield of parasite culture was found to be comparatively much more in semisolid media as compared to that in the other liquid media. Hence, semi-solid medium was preferred over the liquid or biphasic medium for culture and maintenance of *L. donovani* promastigotes (strain UR6).

2. **Biphasic media**: This medium consisted of four parts of salt bactoagar and one part of defibrinated rabbit blood. Positive axenic cultures containing 1-1.5 x 10^6 promastigotes from log to stationary phase were inoculated in each vials or culture flasks asceptically. For contineous maintainance of culture, weekly subculture of parasites or fresh addition of overlay medium was essential. Yield of promastigotes varied considerably in the same medium when different agar base were used. RPMI-1640 medium as overlay and Bacto-agar with 20% defibrinated rabbit blood has been reported to be the most suitable combination for bulk cultivation of promastigotes (Singha, Ph. D. thesis, 1990). This is due to the fact, that bactoagar provides food for promastigotes and at same time it absorbs the motabolic waste products released by promastigotes into the medium (Simpson, 1968).

3. **Liquid media**: The mass cultivation of promastigotes were carried out in liquid media. The promastigotes were inoculated into the medium and incubated at 26 ± 1°C for 4-7 days depending on use of liquid medium. Studies using different medium showed that if same inoculum (1 x 10^6 promastigotes/ml) were inoculated into different medium like L_{15}, RPMI-1640 and M199; then stationary phase culture is reached 4-5 days in L_{15}, 6-7 days in RPMI-1640 and 8-10 days in M 199, respectively. The growth of promastigote was alsofound to be further enhanced by addition of tryptose phosphate broth in L_{15}. Although very rapid multiplication of promastigotes was observed in RPMI-1640 medium but it is some how less as compare to L_{15} medium. Hence

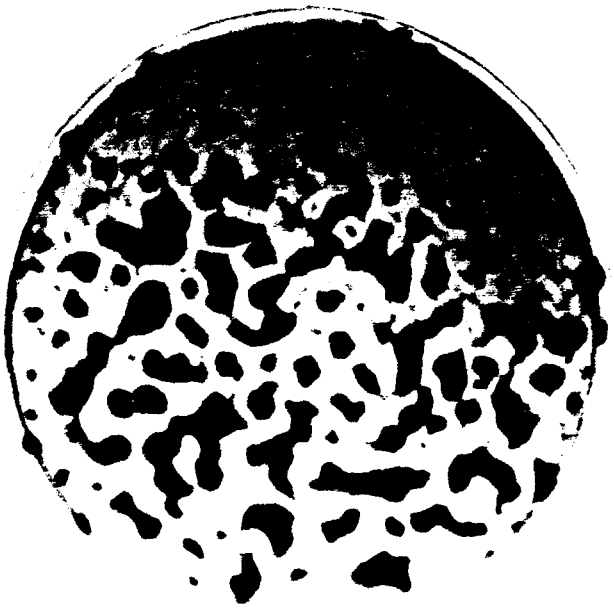
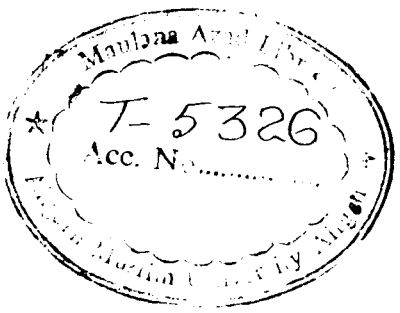


Figure 1: The growth of the *Leishmania donovani* in semi-solid media. All surface an petridish containing the parasites looks like colony or clusters.



L_{15} medium may be used for large scale production of promastigotes. **Plate 2** shows the growth of promastigotes of strain Dd8 in L_{15} medium and promastigotes of strain UR6 in liquid medium.

***In vivo* maintinance of *Leishmania parasites*:**

The parasites were maintained *in vivo* in hamsters through amastigotes to amastigotes by syringe passage or at times by inoculating the stationary phase promastigotes. The amastigotes innoculation has definite advantage over promastigotes inoculation as it takes relatively lesser time for establishment of infection (20 to 25 days for amastigote innoculation and 30-40 days for promastigote innoculation.

The assessment of the intensity of infection was carried out by examining dab smears of spleen; stained with 10% Giemsa in PBS, pH 7.2 for 30 minutes. The slides were exmained under a microscope and classified according to the number of amastigotes/100 cell nuclei.

\+ = 1-10 amastigotes/ 100 cell nuclei
\++ = 11-50 amastigotes/100 cell nuclei
\+++ = 51-300 amastigotes/100 cell nuclei
\++++ = more than 300 amastigotes/100 cell nuclei

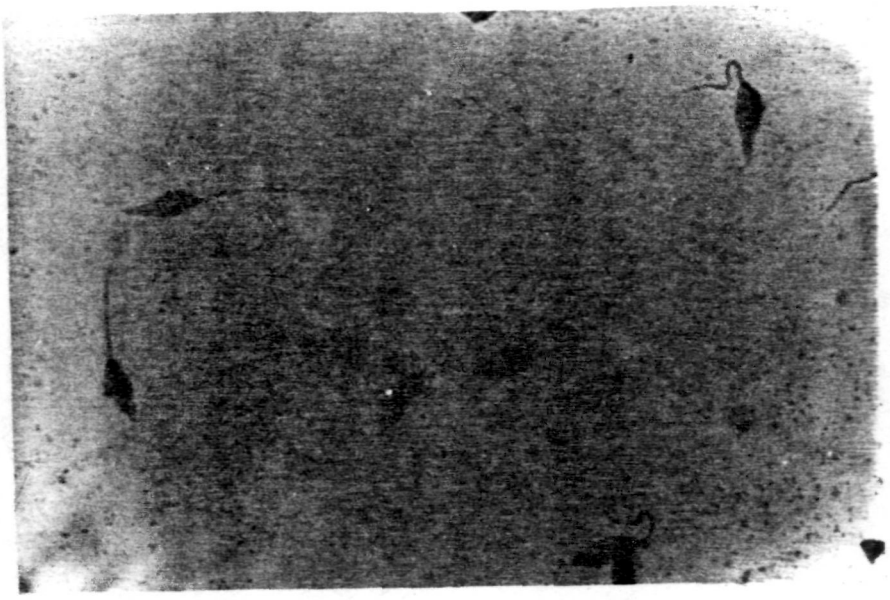
***In vivo* infectivity of *L. donovani* promastigotes of strains Dd8 and UR6:**

In vivo infectivity/pathogenicity of *L. donovani* promastigotes of strains Dd8 and UR6 was examined by inoculating 1 x 10^7 promastigotes in stationary phase of growth to susceptible golden hamsters. Animals inoculated with *L. donovani* Dd8 promastigotes showed a high levels of infection as observed by the high count of amastigotes in the dab smears of spleen on day 45 post inoculation (30-35 amastigotes/100 spleen cell nuclei). Contrary to this, for hamsters inoculated with promastigotes of *L. donovani* strain UR6, not even a single amastigotes in the dab smear of the spleen was observed over an observation period of 180 days post inoculation as in Figure 3. Hence, UR6 strain of *L. donovani* is unable to establish infection in susceptible animals. Hence, non-pathogenicity of *L. donovani* is confirmed as reported by other workers (Chakraborty *et al.*, 1988; Mukherjee *et al.*, 1988; Saha *et al.*, 1986).

GROWTH CURVE:

Since the cell surface molecules and metabolic profile of the various developmental stages of the *Leishmania* promastigotes will be different, hence it is important to study the cell surface molecules of different strains under same developmental stage. Due to this reason growth curve of different strains of *L. donovani* promastigotes under identical conditions were studied. Figure 4 shows the growth curve of promastigotes of Dd8, SS, RMRI and Ag83 strains of *L.*

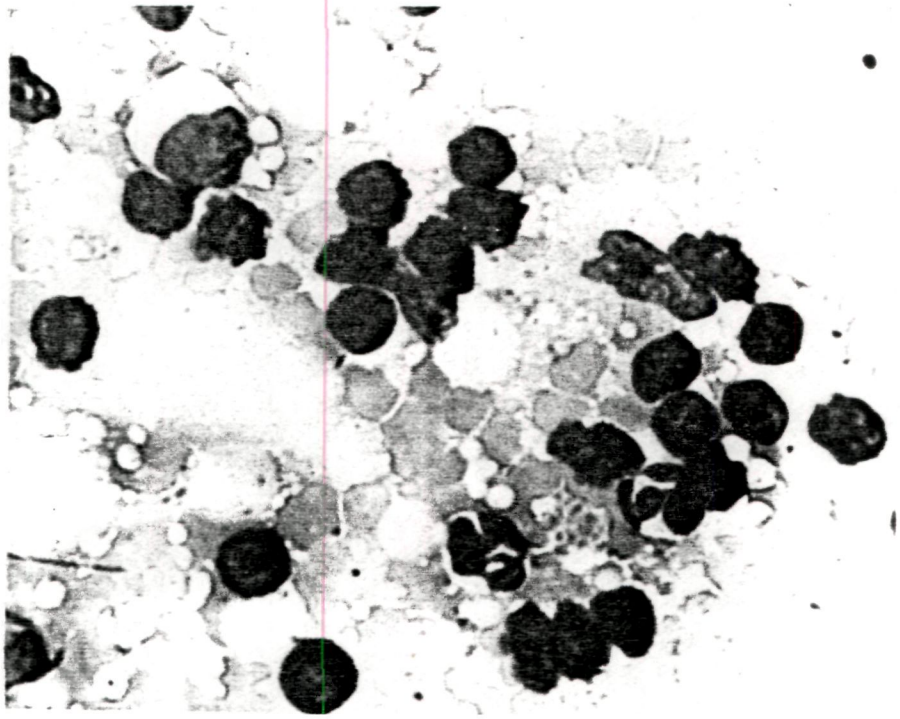
Figure 2: The promastigotes of *L. donovani* growing in liquid media (L_{15}). (a) Promastigotes of Dd8 strain (b) Promastigotes of UR6 strain.



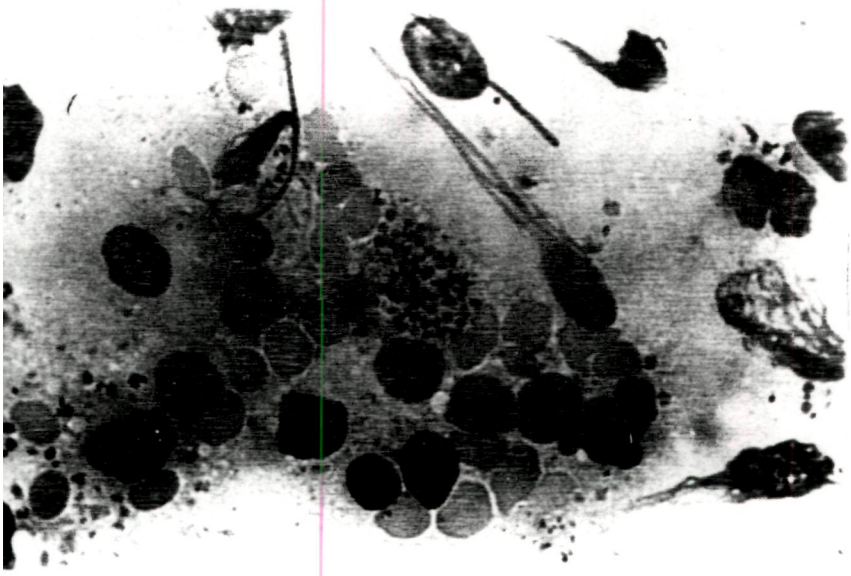
a

b

Figure 3: The Geimsa stained slides of spleen macrophages of *L. donovani* infected Syrian golden hamsters (a) infection from Dd8 promastigotes on day 60 post infection (b) infection from promastigotes of UR6 strain on day 120 days post infection.



a



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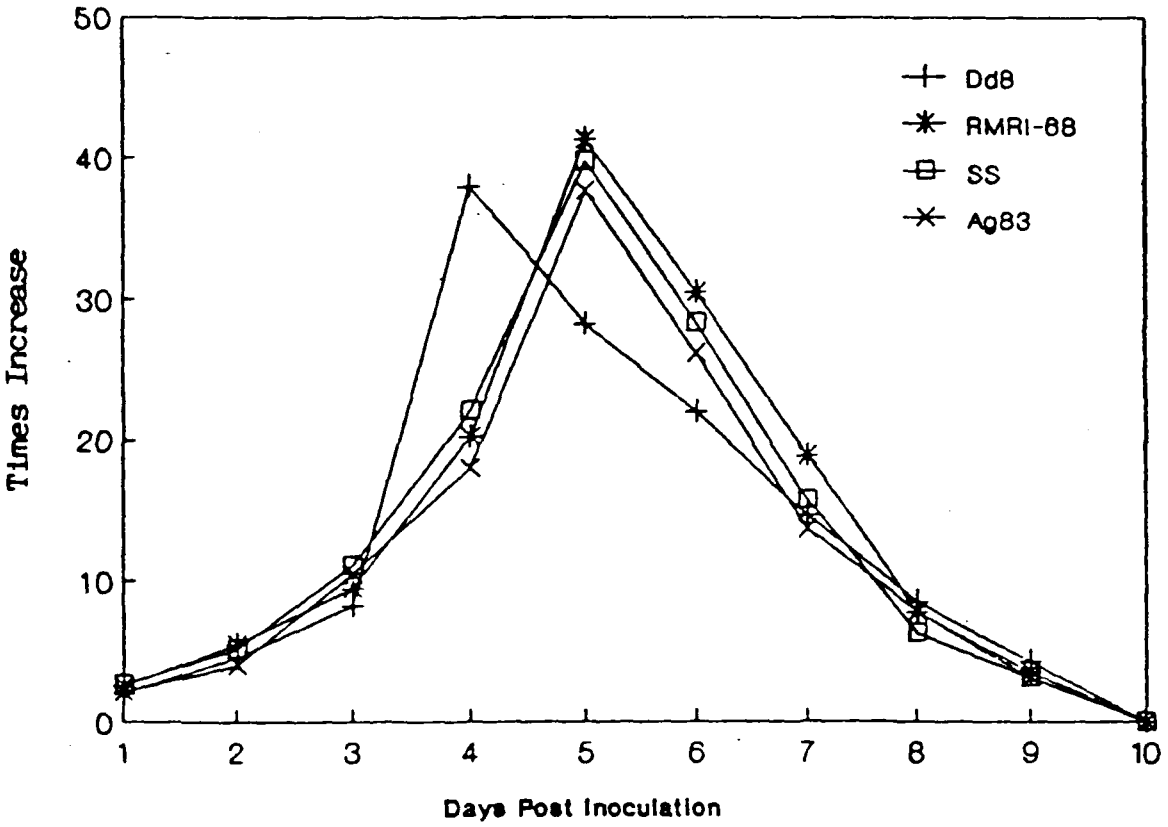


Figure 4: *In vitro* growth profile of *Leishmania donovani* promastigotes in L_{15} medium.

donovani in L_1 medium. Unlike the promastigotes of strains Ag83, SS and RMRI which reach the stationary phase on day 5 post inoculation the promastigotes of strain Dd8 reached the stationary phase on day 4 post inoculation. Hence, the growth of the parasites are depend on species and strain inspite of pH, temperature, nutrients, medium, vectors.

AGGLUTINATION:

The lectin agglutination of *L. donovani* promastigotes of strains UR6, Dd8, RMRI68 was carried out with the four plant lectins Con A, RCA_{120}, PHA-P and PNA, and results are summarized in Figure 5. The Con A concentration causing 50% agglutination were approximately 12 µg/ml, 14 µg/ml and 40 µg/ml for UR6, Dd8 and RMRI strain preomastigotes, respectively. Both, Dd8 and UR6 strains were agglutinated completely with 62 µg ml Con A, while RMRI-68 required about more than 500 µg/ml. The RCA_{120} lectin agglutinated about 50% promastigotes of strains Dd8 and RMRI- 68 with 2 µg/ml whereas 4 µg/ml of lectin was required for promastigotes of strain UR6. 16 µg/ml of RCA_{120} was required for complete agglutanation of all strains of *L. donovani* promastigotes. However, PNA and PHA-P showed different results. 500 µg/ml PHA-P was required for 50% agglutination of promastigotes of Dd8 strain whereas 30% agglutanation was observed for RMRI promastigotes. PHA-:P failed to agglutanate UR6 promastigotes.

The PNA concentrations of 125 µg/ml, 250 µg/ml and 500 µg/ml were require for 50% agglutination of RMRI-68 Dd8 and UR6 strains, respectively. A small proportion of the Dd8 promastigotes (~ 25%) were found agglutinated even at low PNA concentrations (8 µg/ml) whereas the promastigotes of UR6 strain failed to agglutinate 31 µg ml significantly lower (~ 10%) were found to be agglutinate with 125 µg/ml. PNA agglutination was especially inhibited by preincubation with D-galactose suggesting the presence of galactose sugar on cell surface molecules.

Cell agglutination with the various lectins was specific, and no spontaneous aggregation of cells occured in the absence of lectins. Lectin-mediated cell agglutination was inhibited in the presence of specific, competitive lectin binding sugars. Agglutination of the promastigotes found to be lectin concentration dependent (Dwyer, 1977). As demonstrated earlier (Wilson and Pearson, 1984), lectin- agglutinated cells were aggregated randomly, and occurred between all parts of the organisms, i.e. body to flagellum, body to body and flagellum to flagellum, with no preference as to the pole. A number of parasites appeared to agglutinate within themselves as body to flagellum, but in our assay these were counted as free parasites. There was no difference in agglutination at 4°C or 25°C.

Agglutination was specifically inhibited by 10 mM galactose in the case of PNA, and by 10 mM mannose with Con A., RCA-mediated agglutination was not altered by galactose

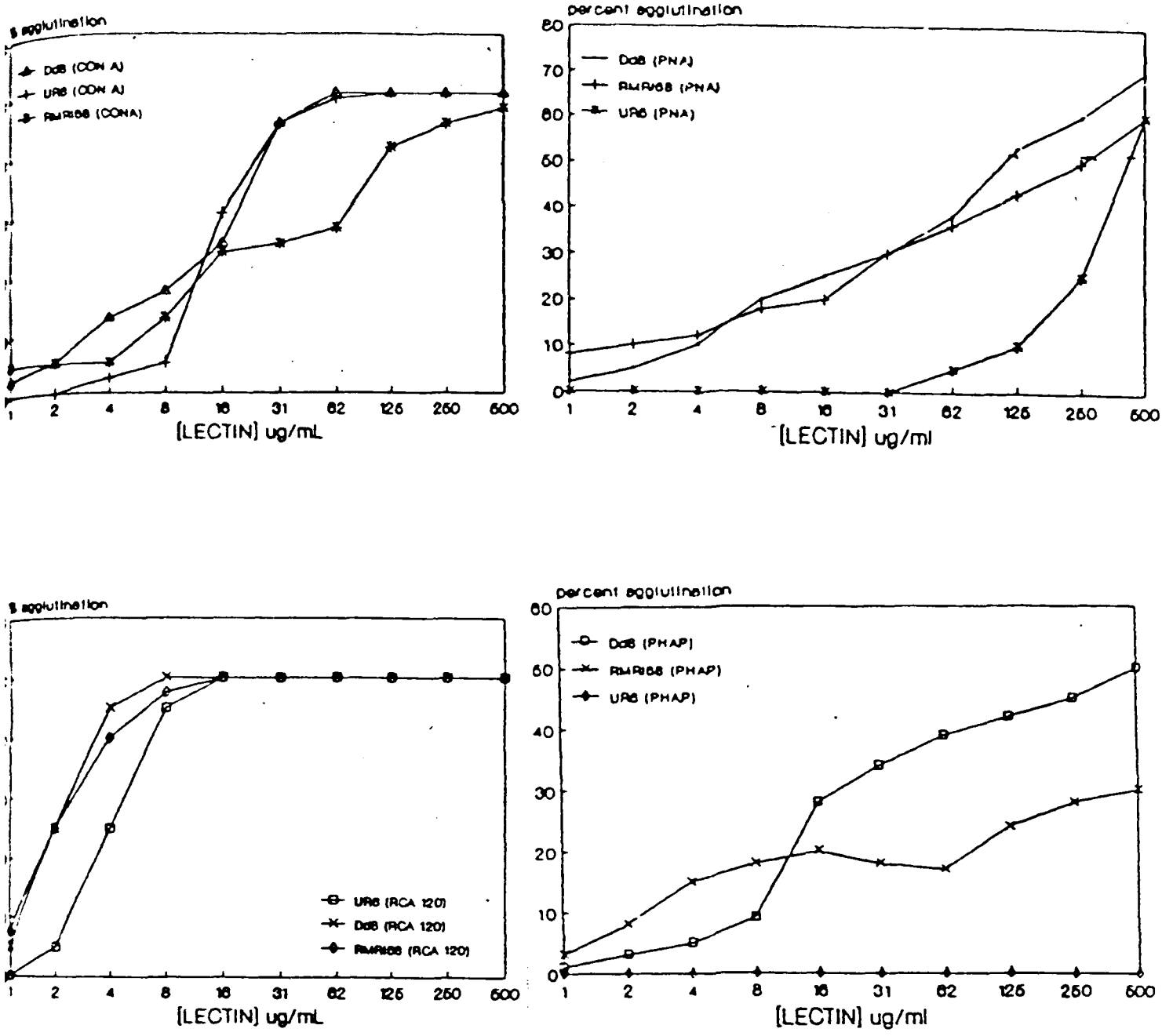


Figure 5: Agglutination profile of *L. donovani* promastigotes of strains UR6, Dd8, RMRI in the presence of four plant lectins Con-A, RCA_{120}, PNA and PHA-P.

but was inhibited by 10 mM lactose. Con A was found to bind to both parasite stages and to agglutinate promastigotes. The carbohydrate binding site of the Con A molecule is known to accommodate both D-mannose and D-glucose residues; inhibition of binding of D-mannose in the case of promastigotes, and by α -methyl mannoside in the case of amastigotes, thus implying that residues containing or resembling mannose were present on the surface of both parasite stages. Similarly, RCA_{120}, which binds to galactose containing saccharides, also bind to both parasite stages, implying the presence of a galactose containing glycoconjugate on the parasite surface (Goldstein and Hayes, 1978; Wilson and Pearson, 1984). The PNA has a binding site that is most complementary to the carbohydrate sequence β-D-galactose (1-->3)-N-acetyl-D-galactosamine with galactose in the penultimate position, and that binds less avidly to D-galactose (Goldstein and Hayes, 1978). Thus in contrast to the RCA binding glycoconjugate(s), PNA may identify an internal galactose rather than one in the terminal position (Wilson and Pearson, 1984).

The structure of the neutral capping sugars on procyclic and metacyclic LPGs suggest that the proportion of various oligosaccharides may expresses β-glalactose, as well as mannose terminating oligosaccharide caps. The metacyclic promastigotes can be purified on the basis of their loss of agglutination by PNA (Sacks *et al.*, 1985), which bind to *L. donovani* promastigotes via recognition of β-linked terminal galactose residues on LPG (King and Turco, 1988). Recently, it was suggested that PNA-negative forms were also ConA negative, and vice versea (Sacks *et al.*, 1995). The structural modifications of *L. major* LPG during metacyclogenesis, are confirmed to the side chain substitutions terminating in β-linked galactose, whereas the predominant Man α1-->2Man capping structure remains unchanged and available for binding by ConA. By contrast, *L. donovani* metacyclics continue to synthesize LPG capping oligosaccharides which are common to procyclic forms, including those containing terminal β linked galactose, α–linked mannose, or both. These residues appear to become cryptic on the surface of metacyclic cells (Sacks *et al*, 1995).

CRYOPRESERVATION OF PARASITES:

Ten percent glycerol was used for cryopreservation. It allows storing of leishmania promastigotes at -60°C for upto 18 months. A gradual decrease in temperature of 1-2°C/30 minutes was carried out before storage in liquid nitrogen. Revival of stored parasite was done by thawing of the vials by rolling them between the palms. A gradual dilution of the sample with culture medium was carried out after thawing, followed by centrifugation and resuspension in fresh medium.

CHAPTER 4

LIPOPHOSPHOGLYCAN OF *LEISHMANIA* PROMASTIGOTE:

ISOLATION, PURIFICATION AND CHARACTERIZATION OF LIPOPHOSPHOGLYCAN FROM NON-PATHOGENIC STRAIN UR6

INTRODUCTION:

Protozoan parasites of genus *Leishmania* causes a number of important human diseases, including visceral cutaneous and mucocutaneous leishmaniasis. During their digenetic life cycle, these parasites alternate between an extracellular promastigote stage in the digestive tract of the sandfly vector and an intracellular amastigote stage that resides within the phagolysosomal compartment of macrophages in the mammalian host. The *Leishmania* parasite has adapted to survive in such hostile environments. There are specialized molecules present on the parasites cell surface which have been implicated for parasites survival. Three antigenic, surface components present in the promastigote stage that play a crucial role have been characterized. These are the surface metalloprotease (gp63) (Bouvier *et al.*, 1985: Etges *et al.*, 1986; Russell and Wilhelm, 1986; Chaudhuri *et al.*, 1989), a family of glycoinositol phospholipids (GIPLs) (McConville and Bacic, 1989; 1990; McConville *et al.*, 1990; McConville and Blackwell, 1991) and a complex glycoconjugate, termed lipophosphoglycan (LPG) (Turco *et al.*, 1984; 1987; Turco, 1988; 1990; Turco and Descoteaux, 1992; McConville *et al.*, 1990; 1992). LPG is the major cell surface glycoconjugate of promastigote stage (Orlandi and Turco, 1987; McConville *et al.*, 1987; King *et al.*, 1987) and is actively secreted into the culture medium *in vitro* (Handman *et al.*, 1984; Ilg *et al.*, 1992). Although LPG is produced by all *Leishmania* spp. (Turco and Descoteaux, 1992; McConville *et al.*, 1992), other parasites have also been shown to express this molecule. Recently, the presence of lipophosphoglycan in *Entamoeba histolytica* (Bhattacharya *et al.*, 1992; Stanely *et al.*, 1992; Prasad *et al.*, 1992; Srivastava *et al.*, 1995) and *Trichomonads* (Singh 1993;1994;Singh and Beach, 1994) has also been demonstrated. LPG molecule was also identified on the cell surface of amastigote stage of *L. major* (Kelleher *et al.*, 1995; Moody *et al.*, 1993; Glaser *et al.*, 1991) and flagellar pocket of *L. donovani* and *L. mexicana* (McConville and Blackwell, 1991; Bahr *et al.*, 1993). LPG plays an important role in the complement activation and resistance to complement mediated lysis (Puentes *et al.*, 1988; 1990; Sacks *et al.*, 1995), in the attachment and entry of promastigotes into mammalian macrophage (Handman and Goding, 1985; Talamas-Rohana *et al.*, 1990; da Silva *et al.*, 1989), in survival in the phagolysosomal compartment (McNeely and Turco, 1990; Elhay *et al.*, 1990) and as a recognition molecule for the T-lymphocyte-dependent immune response characteristic of leishmaniasis (Moll *et al.*, 1989). The promastigotes undergo morphological and biochemical changes within the midgut of the sandfly vector which leads to an increase in their infectivity in the mammalian host (Davies *et al.*, 1990; Sacks *et al.*, 1995). The alteration in pathogenic properties from logarithmic phase promastigotes to stationary phase promastigotes are due to the change in the LPG structure (McConville *et al.*, 1992; Sacks *et al.*, 1995).

In recent years, the promastigote cell surface has received considerable attention in view of its importance for interaction with the immune system and for parasite recognition, uptake and survival in macrophages. the cell surface glycoconjugate, lipophosphoglycan (LPG) has been isolated purified and characterized from *Leishmania* parasites of different species (McConville *et al.*, 1987; Turco *et al.*, 1984; 1987; Ilg *et al.*, 1992). However, no attempt has been made to study the LPG from nonpathogenic strains. Hence, isolation, purification and identification of lipophosphoglycan from a non-pathogenic strain (UR6) of *L. donovani* promastigotes was attempted.

MATERIALS AND METHODS:

ISOLATION OF LIPOPHOSPHOGLYCAN:

Leishmania donovani promastigotes (strain UR6) cells were removed from petri dishes as well as from culture tube by culture wire and suspended in PBS (0.1 M, pH 7.4). The cells were centrifuged at 1500xg for 10 min at 4℃, supernatant was discarded and the pelleted cells were collected. The cells were washed with PBS three times. The parasites were stored at -20℃ in screw-capped antigen vial until use. LPG was isolated using different organic solvent mixutre by the method of Turco *et al.*, (1987).

25 ml of packed cells (27.0 g) were taken in 75 ml of solution having $CHCl_3/CH_3OH$ (3:2, v/v) and 15 ml of 4 mM $MgCl_2$ solution. This was sonicated until the suspension became milky, and then centrifuged at 2000xg for 10 minutes. A pellet in the interface of two phases was obtained. The upper aqueous phase and the lower organic phase containing water soluble metabolites and non-polar lipids, respectively, were discarded. The pellet, containing metabolites, polar lipids, proteins and LPG was collected. To the pellet was added 120 ml of 4 mM $MgCl_2$ solution and sonicated followed by centrifugation at 2000xg for 10 min at 4℃. The supernatant containing metabolites was discarded and again pellet was collected. To this pellet was added 75 ml of $CHCl_3/CH_3OH/H_2O$ (10:10:3, v/v) and 15 ml of $CHCl_3/CH_3OH$ (1:1, v/v) vortexed and sonicated followed by centrifugation at 2000xg for 10 minutes at 4℃. The supernatant containing polar lipids was discarded and to the pellet was added 90 ml of $CHCl_3/CH_3OH/H_2O$ (10:10:3, v/v) solvent mixture vortexed and sonicated followed by centrifugation at 2000xg for 10 min at 4℃. The supernatant was discarded and 90 ml of solvent $CHCl_3/CH_3OH/H_2O$ (10:10:3,v/v) was added to the pellet, it was vortexed sonicated and centrifuged at 2000xg for 10 minutes at 4℃. Supernatant was discarded and to the pellet was added 90 ml of water/ ethanol/ diethylether/ pyridine/ NH_4OH (15:15:5:1:0.017, v/v) sonicated and centrifuged at 2500xg for 15 min at 4℃. Supernatant containing crude LPG was collected in a clean and dried round bottom flask. For complete extraction of LPG, the above step was repeated three times and the solvent extract was combined (288-300 ml). The solvent extract was centrifuged at 30,000xg for 15 min at 4℃

and the supernatant was collected. The discarded pellet contains proteins and glycoproteins. The supernatant was evaporated at 35-40°C and finally lyophilized to get powder form of crude lipophosphoglycan (LPG).

Determination of Carbohydrate Concentration:

For estimating carbohydrate concentration in LPG, Dubois *et al.*, (1956) method was used. Glucose solution, (1.0 mg/ml) was used as standard.

Preparation of Phenol reagent:

Phenol reagent was prepared by adding 20 g of glass distilled water to 80 g of redistilled reagent grade phenol. This 80% phenol solution was diluted to 5% for higher accuracy, hence 1.0 ml of 80% phenol solution was mixed into 15.0 ml of TDW.

0.2 ml of 5% of freshly prepared phenol reagent was added to the 50 µl of crude LPG solution. To this was added 2.0 ml of concentrated sulphuric acid (95.5%). The addition was made by pouring the sulphuric acid against the liquid surface and not at the side of glass tube, for good mixing. The tubes were kept for 10 min at room temperature and then vortexed and incubated at 30°C for 25-30 minutes. Optical density (O.D.) was measured at 485 nm against the blank.

Estimation of Proteins:

The protein contamination in the LPG was analysed by estimation of protein as descibed by Lowry *et al.*, (1951), using bovine serum albumin (free of fatty acids) as standard (1.0 mg/ml).

Preparation of copper reagent:

Copper reagent was prepared by mixing the following reagent:
A solution was made containing 2.0% w/v sodium carbonate in 0.1 M NaOH solution, 2.0% w/v potassium-sodium tartrate and 1.0% w/v copper sulphate in the ratio of (100:1:1, v/v).

Preparation of Folin-phenol reagent:

This is a solution of sodium tungstate and sodium molybdate in phosphoric acid and HCl. Thus the reagent is phosphomolybdotungstate. The commercially prepared reagent was used after dilution with TDW in 1:1 ratio.

Freshly prepared copper reagent 5.0 ml was added to 50 µl of sample solution (brought to 1.0 ml by addition of distilled water). This mixture was incubated for 10 minutes

at room temperature. then 0.5 ml of diluted folin Phenol reagent was added to each tube. The tubes were shaken vigorously. After 30 minutes. the colour intensity of the reaction was read at 750 nm, using 160 UV/ visible Simadzu spectrophotometer.

PURIFICATION OF LPG:

Gel filtration Chromatography:

Packing of the column is the critical factor in achieving a optimum separation of any mixture by column chromatography. A clean and dried column was pluged by glass wool. Packing of a column was normally carried out by gently pouring a slurry of the stationary phase (resin or gel) into a column after removing fines' of the gel. The column was gently tapped to ensure that no air bubbles are trapped and the packing settles evenly. The slurry was added until the required height was obtained. Once the required height was obtained, the flow of the solvent through the packed column was started and continued until the packing was completed. The viod volume and retention volume of the column was checked.

The purification of crude lipophosphoglycan (LPG) was carried out by column chromatography. The void volume and retention volume of the column were measured using blue dextran and phenol red, respectively. The crude LPG was dissolved in 2.0 ml of 40 mM NH_4OH containing 1 mM EDTA and centrifuged at 9000xg for 3 minutes. The supernatant was collected and applied on Sephadex G-200 column (1.45 cm x 60.0 cm), equilibrated with 40 mM NH_4OH containing 1.0 mM EDTA. The fractions (1.0 ml) were collected and analysed for carbohydrate by the method of Dubois *et al.*, (1956). The protein contamination in the partially purified LPG, was checked by the method of Lowry *et al.*, (1951). The fractions containing LPG were pooled and dried by lyophilization. The completely dried LPG was then resuspended in 1.0 ml of 40 mM NH_4OH and loaded on Sephadex G-25 column (1.3 x 45.0 cm) equilibrated with the same buffer at flow rate 30.0 ml/hour for desaltation. The LPG containing fractions were collected and lyophilized (Orlandi *et al.*, 1987).

The ammonia containing solution does not cleave acyl linkage, therefore. the dried LPG was resuspended in 10.0 ml of water/ethanol/diethylether/pyridine/NH_4OH (15:15:5:1: 0.017, v/v) and was precipitated by addition of equal volume of methanol and chilling the sample at -20°C for 18-20 hours. After methanol precipitation solvent and methanol were evaporated under vacuum (Orlandi *et al.*,1987).

Proteinase K treatment:

Dried LPG was treated with proteinase K enzyme for removal of tightly bound or associated protein to LPG (Ilg *et al.*,1992). Dried LPG was dissolved in 2.0 ml of Tris-HCl buffer

(10 mM, pH 8.0) and added 100 µg/ml proteinase K enzyme. The solution mixture was incubated at 42°C for 3-3.5 hr. After the treatment, LPG was extracted by centrifugation at 5000Xg for 10 min at 4°C. Then, the aqueous phase was extracted with one volume of phenol saturated with 10 mM Tris-HCl buffer, pH 8.0.The phenolic phase was twice extracted with one volume of 10 mM Tris-HCl, pH 8.0. The bulk of the phenol in the combined aqueous phase was extracted twice with chloroform and 3-methyl-1-butanol (24:1,v/v). It was concentrated by rotatory evaporation about 1/3 volume and ethanol precipitation was performed. The precipitation was carried out by addition of 1/10 volume of 5M ammonium acetate and 4 volume of ethanol and incubated for 3 hr at -70°C, precipitate was collected by centrifugation and dried under reduced pressure.

Hydrophobic chromatography:

A small column was washed dried and packed with phenyl Sepharose CL-4β or octyl Sepharose CL-4B gel as described earlier. Preferably octyl Sepharose CL-4B was used as it is more hydrophobic than phenyl Sepharose CL-4B. The loading of the sample was carried out at room temperature with high salt concentration so as to ensure complete binding, while elution of the material was performed at low temperature. high pH and reduced salt concentration for high recovery of the bound materials.

Finally, dried LPG was resuspended in 1.0 ml of 0.5 M NaCl and 0.5 M acetic acid and applied onto octyl Sepharose column (1.5 x 10 cm). The column was initially equilibrated with 0.1 M NaCl in 0.1 M acetic acid. After 20 fractions(1.0 ml tube) were collected, the solution was changed to 0.1 M acetic acid where 20 more fractions were collected. Finally column was washed with TDW.

The lipid portion of LPG gets bound to the octyl or phenyl Sepharose due to hydrophobic interactions. Leaving PG and other carbohydrates to be eluted out. The bound LPG was eluted with solvent E (Turco *et al.*,1987) or a gradient 5- 70% propanol in 100 mM, TES buffer, pH 7.0 (Ilg *et al.*, 1992). All fractions were analysed by the method of Dubois *et al.*, (1956). LPG containing fractions were pooled, dialyzed against distilled water and lyophilized.

IDENTIFICATION OF LPG:

Thin Layer Chromatography (TLC):

The purified LPG was identified by thin layer chromatography (TLC). The analysis of LPG was carried out on silica gel-G precoated plate. The principle of separation on TLC is that, the molecules interact with both the support of silica gel and the solvent system. LPG was

dissolved in solvent E; water/ethanol/diethylether/pyridine/NH_4OH (15:15: 5:1:0.017, v/v). 5-10 µl of this was applied on the TLC plate and dried at room temperature. The plate was run in a solvent mixture n-propanol/pyridine/water (1:1:1, v/v) till the solvent reached the top of the plate. The plate was taken out from the chamber, and dried at room temperature and then in the oven. This was stained with iodine followed by 1.0% resorcinol in 10% sulphuric acid (Orlandi *et al.*, 1987 and Turco *et al.*, 1987). The TLC plate was heated in oven 5-10 minutes as to cause charring of carbohydrate moieties of LPG.

Preparation of Schiff's Reagent:

Schiff's reagent was prepared (Korn *et al.*, 1973) by addition of 2.5 g of basic fuchsin in 500 ml of distilled boiling water. Stirred for 30 minutes and cooled to 50°C and then filtered. 40 ml of 1.0 N HCl was added to the filtrate, cooled to 25°C and then 3.5 g sodium metabisulphite was added. This was kept in the dark for 20 hours at 4°C. 2-3 g of activated charcoal was added and shaken vigorously, filtered and the pH was adjusted by adding 1N HCl until a drop when dried on the glass slide does not turn red. This was stored at room temperature. The solution has to be diluted with TDW (1:1,v/v) before use.

Procedure for PAS staining:

The gel was transferred in 12.5% TCA for 60 min in dark and cool place. It was rinsed for 5-10 min in TDW and immersed in 1% periodic acid containing 3% acetic acid solution and incubated for 1.0 hr in dark and cool place. The gel was washed thoroughly at 4°C with TDW with intermittent shaking and was left overnight in TDW after at least 6-8 changes of fresh water. The gel was transferred into fuchsin- sulphite in dark for 30-45 min for staining and then washed 3-5 times with 0.5% sodium-metabisulphite in 3% acetic acid solution. Finally, the gel was washed with H_2O according to requirement and was stored in 3-5% acetic acid solution.

Preparation of Silver stain:

Silver stain was prapared as per the method of Ookley *et al.*, (1980). All solutions to be prepare fresh.

(i) 0.36% (W/V) NaOH in H_2O

(ii) 1.0 ml ammonia solution (25%) plus 1.05 ml H_2O

Solution (A): mixed 21.0 ml of solution (i) and 1.4 ml of solution (ii)

Solution (B): 1.0g $AgNo_3$ in 5.0 ml H_2O

Solution (C): Added solution (B) drop by drop to solution (A) in a beaker with continuous

stirring. The addition of solution (B) was stopped on appearance of brownish colour or an insoluble precipitate. If precipitate was of much higher amount then it has to be prepared fresh, otherwise, it is transferred to a measuring cylinder and made upto 100 ml and used immediately.

Solution (D):(i) 1.0% citric acid (w/v) in H_2O

(ii) 38.0% HCHO in H_2O

2.5 ml of (i) and 0.25 ml of (ii) were added in 500 ml H_2O and used for development of band.

Procedure:

The slab gel was fixed in 50% methanol and 10% acetic acid solution for 3-4 hr or over night. It was transferred in 5% methanol, 7% acetic acid solution for 1hr and washed with TDW for 30-45 min or more, so that the gel recovers its normal size. The staining solution (C) was added in the plastic box containing gel. This box was then placed on to the shaker for 30 min for proper staining. This gel was washed in the H_2O for 5-10 min with gentle shaking. The stained gel was transferred in the developer (D) and was shaken gently until the bands were visualized. Finally, the gel was washed 3-4 times with H_2O and stored in 5% acetic acid solution.

Coomassie brilliant blue stain:

0.5 g Coomassie brilliant blue R-250 was dissolved in 450 ml of methanol and stirred it for 30 min. Then 100 ml, of acetic acid was added and made the final volume to 1.0 liter and stored at room temperature.

Sodium Dodecyl Sulphate Polyacrylamide gel electrophoresis (SDS-PAGE):

For determination of molecular weight of LPG, SDS- polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Guilian *et al.*, (1983; 1985). The separating gel (pH 9.3) was(15% T, 0.5%C) and the stacking gel was (5% T, 4.8% C) generally used. LPG was dissolved in sample buffer to give a final concentration of 0.1% (w/v) SDS, 15% (v/v) glycerol (pH 6.8) and a trace amount of bromophenol blue as a tracking dye and dithiothreitol (DTT) was used. The sample was centrifuged for 4 minutes at 20,000xg. 30 µl (1 mg/ml) of sample was loaded in a well and electrophoressed at 15 mA constant current. Running buffer containing 0.38 M glycine, 0.05 M Tris and 0.1% SDS (pH 8.5) was used. The molecular weight markers of range 66 kDa-14.1 kDa were run for determination of molecular weight. Coomassie brilliant blue and periodic acid Schiff's (PAS) staining were carried out for detection of protein and LPG, respectively (Korn and Wright, 1973;

Orlandi *et al.*, 1987). The gel was destained in 45% methanol, 10% acetic acid for protein and 0.5% sodium metabisulphite in 3.0% acetic acid for carbohydrate analysis of LPG.

One dimensional ^1H and 2D COSY Nuclear Magnetic Resonance spectroscopic studies:

Samples were prepared for NMR studies by repeated dissolution in 99.98% D_2O with evaporation and lyophilization. Finally, the sample was dissolved in 500 µl of D_2O (Aldrich). 1D and 2D COSY-^1H NMR experiments were carried out at room temperature (25 C) on a Bruker WM 400 NMR spectrometer equipped with ASPECT 2000 computer using a $^{13}C/^1H$ 5 mm dual probe head. LPG was taken in D_2O (99.98%) with sodium-2.2- Dimethyl-2-silapentane-5-sulfonate (DSS) as a reference standard. Typical Fourier Transformation conditions for one dimensional ^1H NMR were as follows. Sweep width (SW) was 6400, size 16k, Pulse width (PW) = 45° with a digital resolution of 0.77 Hz per point.

The two dimensional (2D) COSY experiment used was N-type phase cycling and a 90° mixing pulse. The free induction decays were acquired over 1024 data points and 2463.05 Hz for each of 256 values of evolution time. The second dimension (F_1) sweep width (SW_1) was 1058.8 Hz. The raw data were zero filled in both dimensions prior to double Fourier Transformation.

RESULTS:

Isolation of LPG:

LPG from UR6 strain was extracted essentially by the method of Turco *et al.*, 1984; 1987, using different organic solvent mixture like, $CHCl_3/CH_3OH$ (3:2, v/v), $CHCl_3$ / CH_3OH/ H_2O (10:10:3, v/v), $CHCl_3$/ CH_3OH (1:1, v/v) and water/ethanol/ diethylether/pyridine/ammonium hydroxide (15:15:5:1:0.017, v/v) solvent E followed by sonication and differential centrifugation. When the parasite cell suspension was treated with chloroform and methanol various metabolites, polar and non-polar lipids were extracted. Delipidated residue which was obtained in the form of pellet was extracted efficiently at 4°C with solvent E which contains water /ethanol / diethylether/ pyridine/ ammonium hydroxide in a proportion of 15:15:5:1:0.017. Various solvents have been tried for solubilization of delipidated materials. It has been observed by Turco *et al* (1984) that about 95-97 glycoconjugate is extracted from delipidated material with 40 mM NH_4OH at 100°C, while only 85-90% was extracted at 25°C with solvent E. Hence, considering this fact low temperature and optimum pH is required to extract cell surface LPG's inorder to prevent its degradation. Thus, solvent E was preferred over NH_4OH. This fact is also supported by various other workers including (King *et al.*, 1987; Orlandi and Turco, 1987, Jaffe *et al.*, 1990).

In the isolation of LPG from UR6 promastigotes from 25.0 ml (27.0 gm) of packed cells (promastigotes) about 150 mg of crude LPG was obtained. The presence of carbohydrates in crude LPG was determined by phenol-sulphuric acid method (Dubois *et al.*, 1956). Figure 1 shows the presence of carbohydrates in absorbance spectrum of crude LPG in the wavelength region of 450-550 nm. The absorbance maxima (λ_{max}) was observed at 480 nm, which indicated the presence of carbohydrates in the extracted LPG sample. The broad peak of crude LPG was indicative of the fact that heterogeneous population of carbohydrates was present in the sample. When the crude LPG sample was subjected to SDS-PAGE, it migrated as a broad band with an approximate molecular weight range between 35-10 kDa. PAS staining did not reveal presence of any other glycoconjugate in this extract. Coomassie brilliant blue R-250 staining of SDS-PAGE gels showed dye positive bands indicating that the extract was contaminated with a number of proteins.

PURIFICATION OF LPG:

The partial purification of crude LPG was carried out by size exclusion chromatography. As reported earlier, gel filtration of glycoconjugate on Sephadex or Sepharose column equilibrated with 0.2 M acetic acid, 0.1 M NH_4HCO_3, 0.1 M Na_2CO_3 or 0.1 M Tris-HCl, pH 8.0, resulted in poor recovery of the applied materials on column. Purification of the glycoconjugate on Bio-gel support or Sepharose equilibrated with any of above mentioned buffers or with 0.04 M NH_4OH and 0.001 M EDTA solvent failed to elute the material quantitively. Therefore, it is suggested that glycoconjugate is purified efficiently on a Sephadex gel equilibrated with 0.04M NH_4OH containing 0.001 M EDTA solution. Sephadex G-200 gel was prefered over Sephadex G-100 (Turco *et al.*, 1984; 1987; King *et al.*, 1987) due to its high resolution and coverage of maximum range for separation. NH_4OH solvent was useful for avoiding the precipitation of the LPG on the top of the column. Addition of the EDTA in the eluting solvent was necessary to prevent aggregation of LPG, as without EDTA, LPG eluted much earlier as an aggregate on gel-filtration.

The crude LPG was dissolved in 2.0 ml of a solution containing 40 mM NH_4OH and 1.0 mM EDTA. The particulates material was removed by centrifugation and loaded (4.6 mg/ml carbohydrates as determinedby Dubois *et al.*, 1956) on Sephadex G-200 column equilibrated with same buffer. The elution profile of LPG from Sephadex G-200 is shown in Figure 2. LPG fractions eluted just after void volume, indicating that it is a high molecular weight molecule. Single broad peak was obtained indicating the heterogeneous nature of LPG. The desaltation of LPG molecule which is eluted from Sephadex G-200 was necessary for removal of unwanted salts like, EDTA, NH_4^+, Mg^{++} etc. (King *et al.*, 1987). Desalting of LPG sample was carried

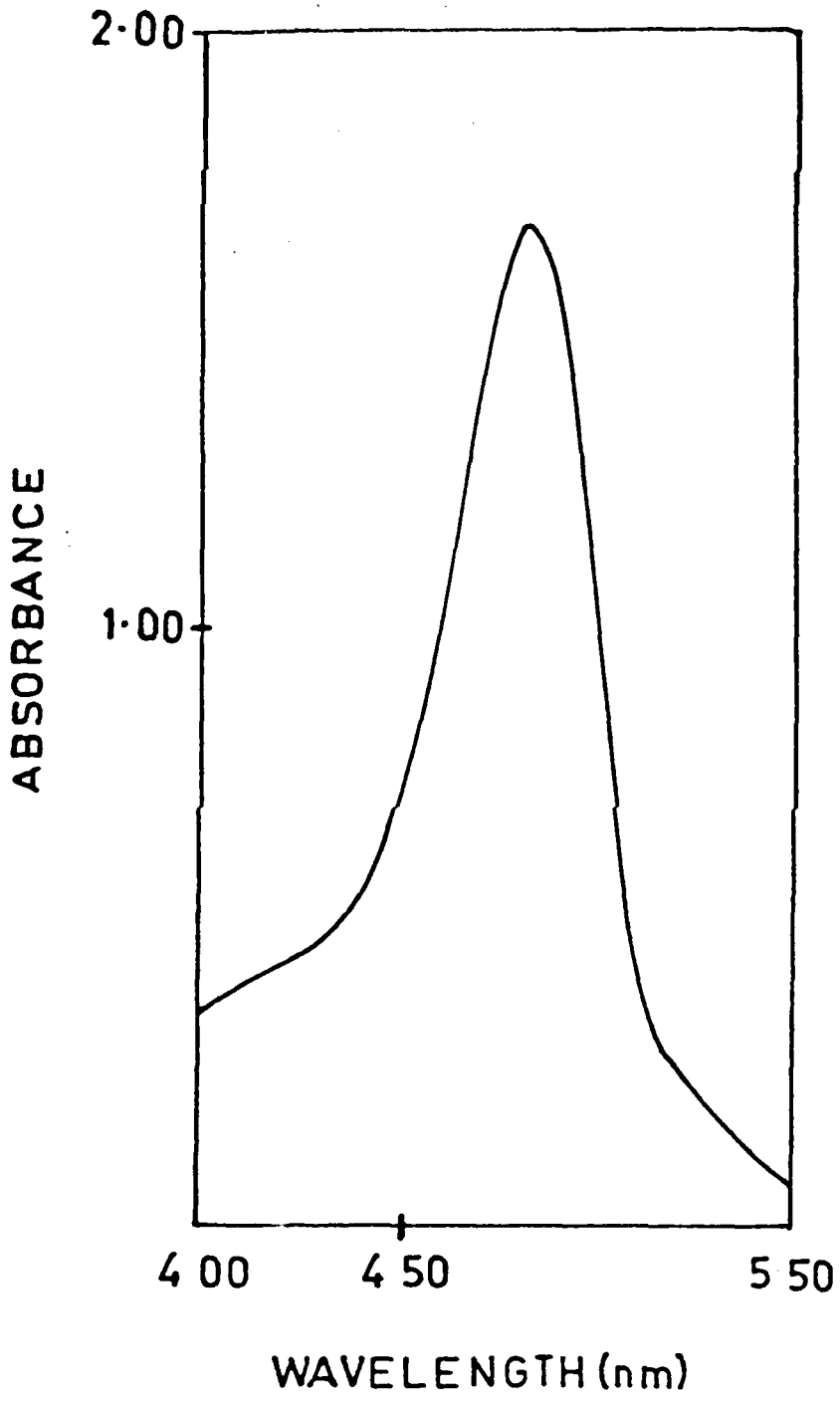


Figure 1: The absorbance spectra of crude LPG on reaction with phenol-H_2SO_4 reagent (Dubois *et al.*, 1956). The absorbance maxima was observed at 480 nm, which indicated the presence of the carbohydrates.

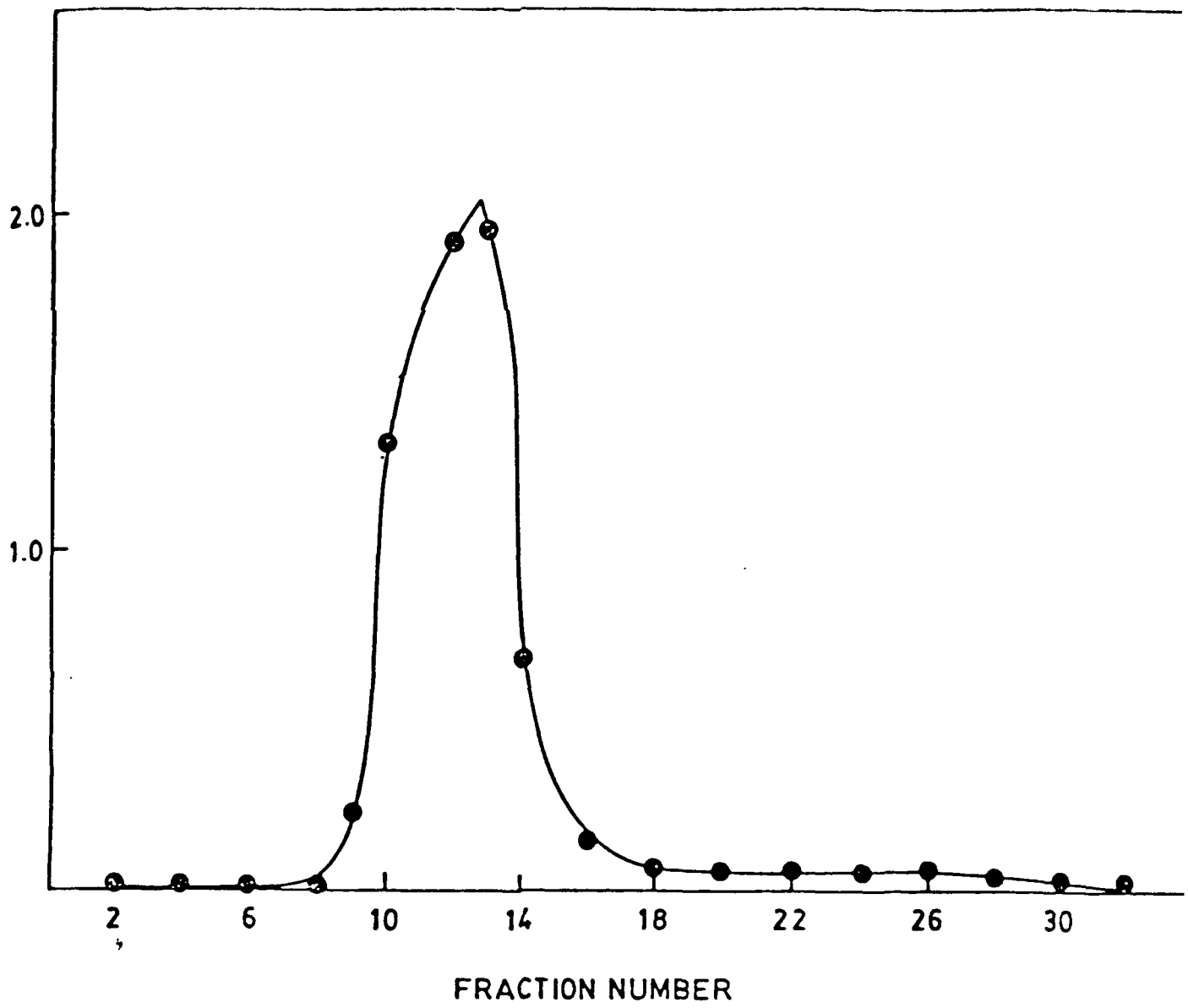


Figure 2: Elution profile of LPG on Sephadex G-200 column (1.5 x 20 cm). 2.0 ml sample in 40 mM NH_4OH containing 1.0 mM EDTA was loaded. Flow rate of the column was 4 ml/hr and 0.8 ml fractions were collected. Void volume of column was 7.0 ml. Fractions were monitored by the method of Dubois *et al* (1956).

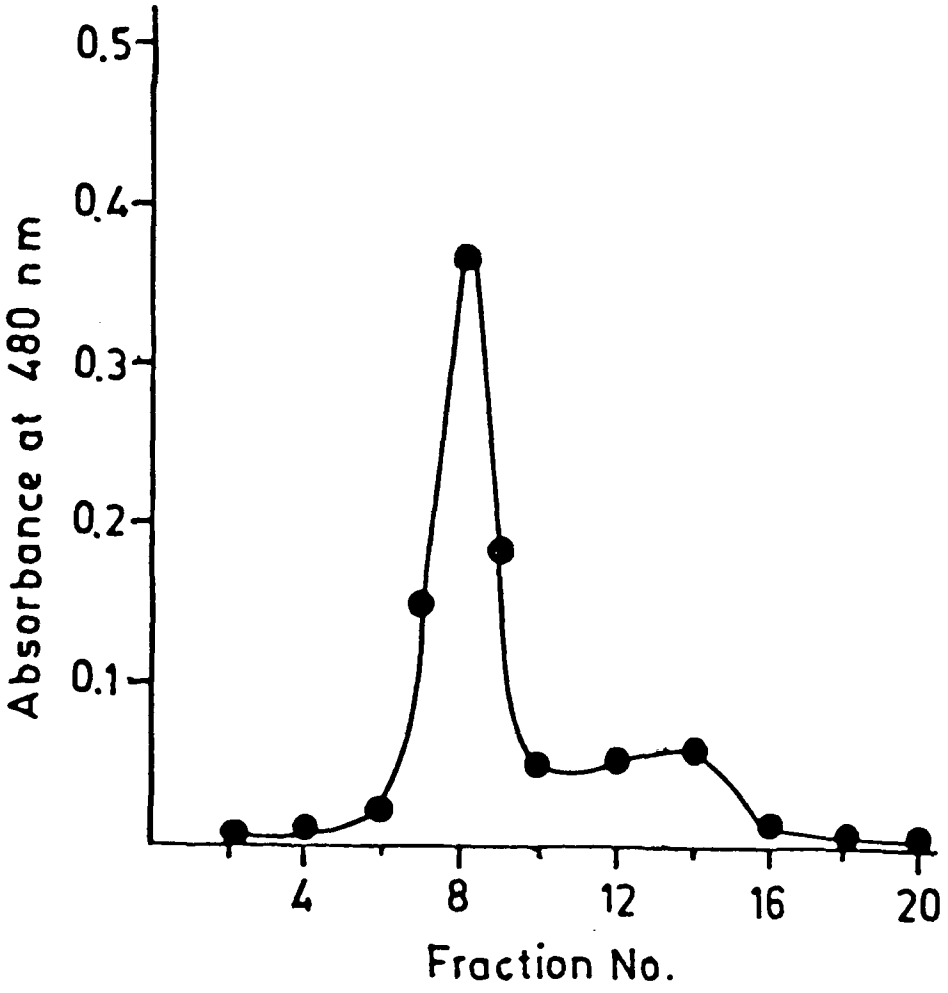


Figure 3: Elution profile of LPG on Sephadex G-25 column (1.7 x 10 cm). Flow rate of the column 36 ml/hr and 1.0 ml fractions were collected.

out on Sephadex G-25 column. It is necessary to remove EDTA from the LPG sample, as it interfered in the NMR studies. The elution profile of Sephadex G-25 column is shown in Figure 3. A single broad peak was again obtained, which indicated single type of population. Protein contamination in the LPG sample was not detected by conventional methods such as, Ninhydrin test, Lowry method and coomassie brilliant blue-R-250 staining on gels. The absence of proteins, DNA and RNA was confirmed by UV-spectroscopy where the LPG preparations showed no absorbance at 280 nm and 260 nm respectively. However, with ^1H NMR and silver staining, protein contamination was observed. Therefore, further purification of LPG molecule was essential. Thus, the LPG smaples were treated with proteinase K as described in materials and methods for removal of tightly associated proteins/peptides. Proteinase K treated LPG fraction was applied on octyl Sepharose CL-4B column for final purification. The LPG samples recovered from the column did not show any silver stain positive band on SDS-PAGE gel, suggesting that proteinase K treatment of LPG sample renders it free of any protein contamination. The final confirmation of the absence of protein contamination was carried out by ^1H NMR spectroscopic analysis. No peak corresponding to -NH signal of the protein was observed. Hence, pure LPG was obtained, which was used for further studies.

From 25.0 ml (27.0 gm) packed cells, about 150 mg of crude LPG was obtained. On further purification by column chromatography, 88.0 mg of partially purified LPG from Sephadex G-200 and 31.0 mg from Sephadex G-25 was obtained. After removal of tightly associated protein contaminants and final purification by hydrophobic chromatography, 15.5 mg pure LPG was obtained.

IDENTIFICATION OF LPG:

Identification of purified LPG was carried out by thin layer chromatography (TLC), SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and ^1H NMR spectroscopy. Purified LPG was dissolved in solvent E and applied on silica gel G precoated plates. Figure 4 shows the mobility of LPG in the solvent mixture 1-propanol/pyridine/H_2O (1:1:1, v/v). A single diffuse spot, characteristic of LPG molecules (Orlandi and Turco, 1987), was observed. The R_f value of LPG in this solvent system was found to be about 0.66 ± 0.02, which is close to 0.62 reported earlier for the *L. donovani* promastigotes LPG of pathogenic strain (Orlandi and Turco, 1987).

Purity of LPG molecule was also assessed by SDS-PAGE. The SDS-polyacrylamide gel electrophoresis followed by periodic acid schiff's (PAS) staining and coomassie brilliant blue R-250 and silver staining of purified LPG are shown in figure 5. As seen in Figure (5A), no protein contaminants were observed on coomassie or silver staining of SDS-PAGE of finally purified LPG, thus indicating that the purified UR6 LPG was devoid of any protein contaminant.

Figure 4: Thin layer chromatography (TLC) of purified LPG. An aliquot (5-10 µl) of the LP sample was chromatograph on silica gel G in solvent (propanol/pyridine/H_2O, 1:1 v/v). Detection was carried out by charring with 1.0% resorcinol in 10% sulphuric ac

Lane 1 : Sucrose, Lane 2 : Pure LPG, Lane 3 : Crude LPG

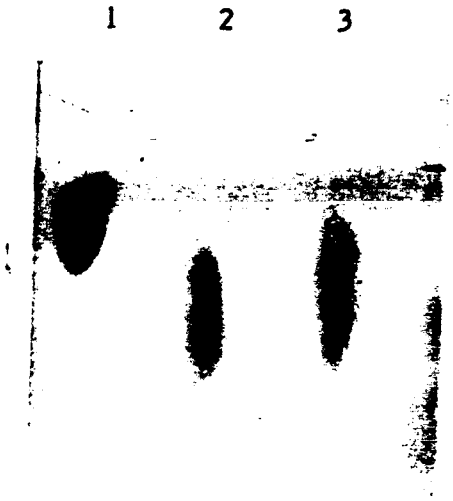
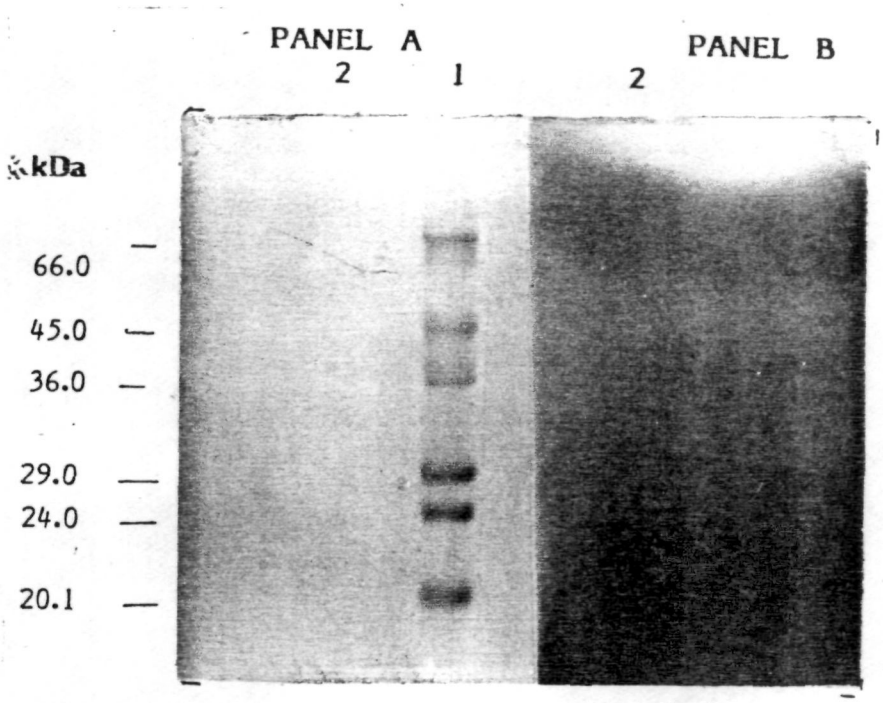

1
2
3

Figure 5: SDS-Polyacrylamide gel profile of LPG purified from *L.donovani* promastigotes non-pathogenic strain UR6. Shown in Figure A; Panel A is a coomassie brilliant blu R-250 and Panel B is a periodic acid-Schiffs base stained gel. Figure B; Panel is silver stained and Panel B is a periodic acid-Schiffs base stained gel.
Lane 1 is molecular weight marker , lane 2 is purified LPG and lane 3 is partial purified LPG.

a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1
PANEL B
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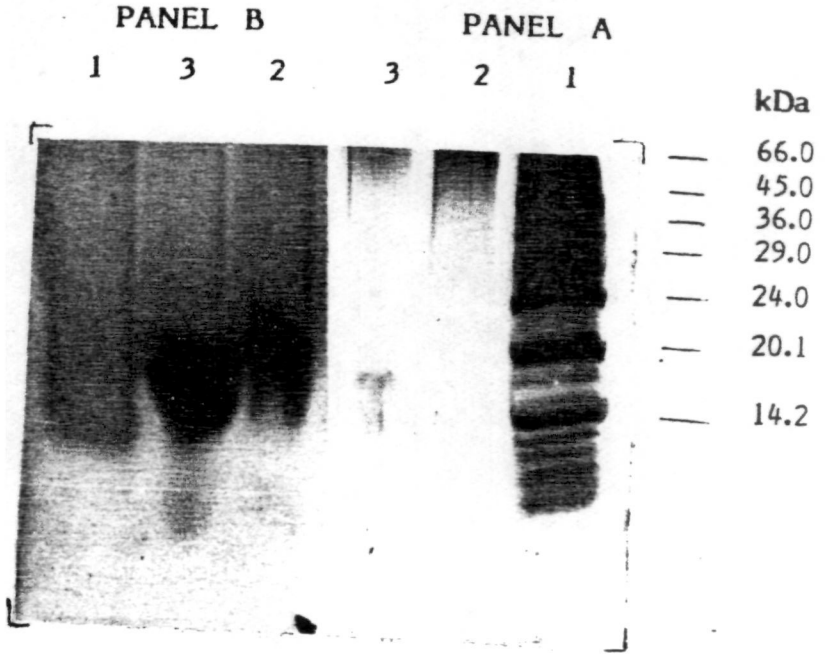
20.1

b


PANEL B
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14.2

Tightly associated protein contaminants which were observed as positive bands on silver staining of LPG molecule on partial purification from Sephadex G200 were removed by proteinase K treatment. On PAS staining of gel, LPG was the only carbohydrate staining species observed and it migrated as a broad band. The molecular weight of UR6 LPG, obtained by the migration of standards protein on SDS-PAGE, as shown in Figure (5B) was found to be in the range of 20-25 kDa. The observed molecular weight of UR6 LPG is similar to that reported earlier for LPG of promastigote of *L. donovani* (Turco *et al.*, 1984; 1987).

^{1}H NMR spectroscopic studies were carried out to determine the structural features of LPG. 400 MHz ^{1}H NMR spectrum of LPG molecule is shown in Figure 6. The signal at δ5.52 ppm corresponds to the H-1 proton of mannose. The signal at δ 4.94 ppm from the HOD and the signal from δ4.3 to 4.54 ppm correspond to anomeric protons of carbohydrate moieties. The doublet at about δ1.0 ppm is of the terminal CH_3 of acyl chain of lipid moiety and that at δ1.3 ppm corresponds to the $(CH_2)_n$ of the acyl chain of lipid moiety.

Two dimensional COSY NMR studies were carried out for structural characterization of the LPG molecule. A two dimensional counter plot (COSY), below the normal one dimensional spectrum is shown in Figure 7. The signal at δ 5.52 ppm was unambiguously assigned to H-1 of mannose and connectivities could be traced out as cross peak to the signal at δ 3.71 ppm for H-2 protons, respectively. The H-2, signal further gave cross peak at δ3.53 for H-3 proton. Further assignments of other proton in mannose was difficult in the region δ 4.0-3.35 ppm due to the in phase properties of diagonal peak. Furthermore the presence of three anomeric protons at δ 4.47, 4.26 and 4.1 ppm indicated that LPG was a very complex structure. The signal at δ 4.47 can be unambiguously assigned to H-1 proton of galactose. Similarly connectivities could be traced out for H-2 (δ3.54 ppm), and H-3 (δ3.83 ppm), but due to the aforesaid problems further connectivities could not be traced out.

DISCUSSION:

The lipophosphoglycans of *L. donovani* (strain UR6) are a heterogenous family of molecules which having a tripartite structure, consisting of variable disaccharide phosphorylated repeat units, phosphosaccharide core and a lysoalkyl-PI lipid moiety. As reported earlier, the glycoconjugate is removed easily from a delipidated residue fraction with several solvent at 100°C, but extraction with solvent E at 25°C or less is preferred. The use of solvent E is desirable during extraction of LPG at lower temperature and optimum pH for avoiding its degradation. Solubility of the LPG in solvent E which contains ethanol, ether, and pyridine suggested that the LPG possesses hydrophobic regions. This possibility was further strengthened by observation that

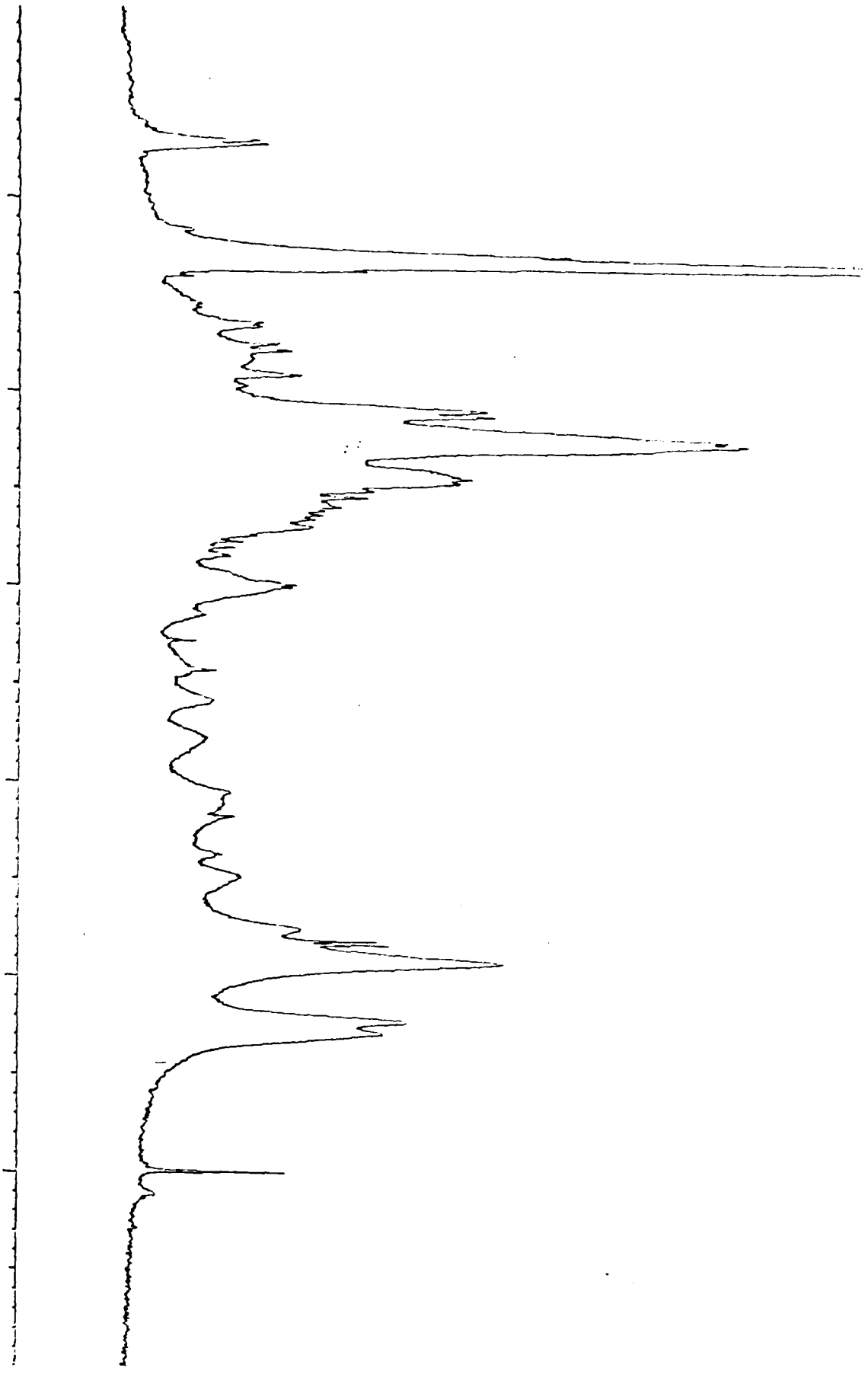
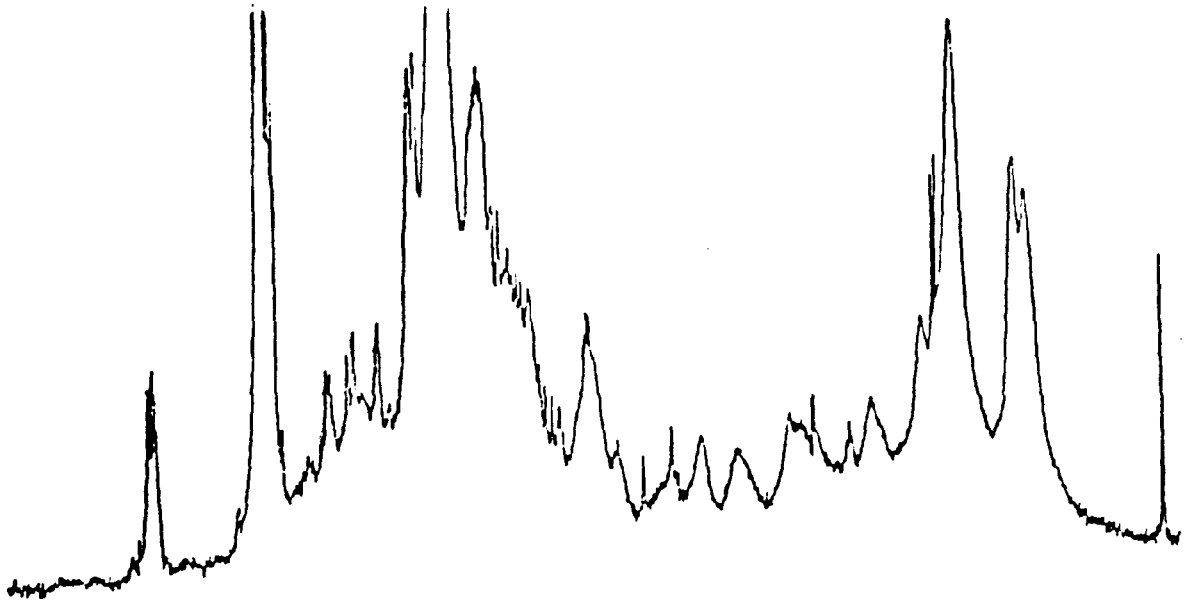
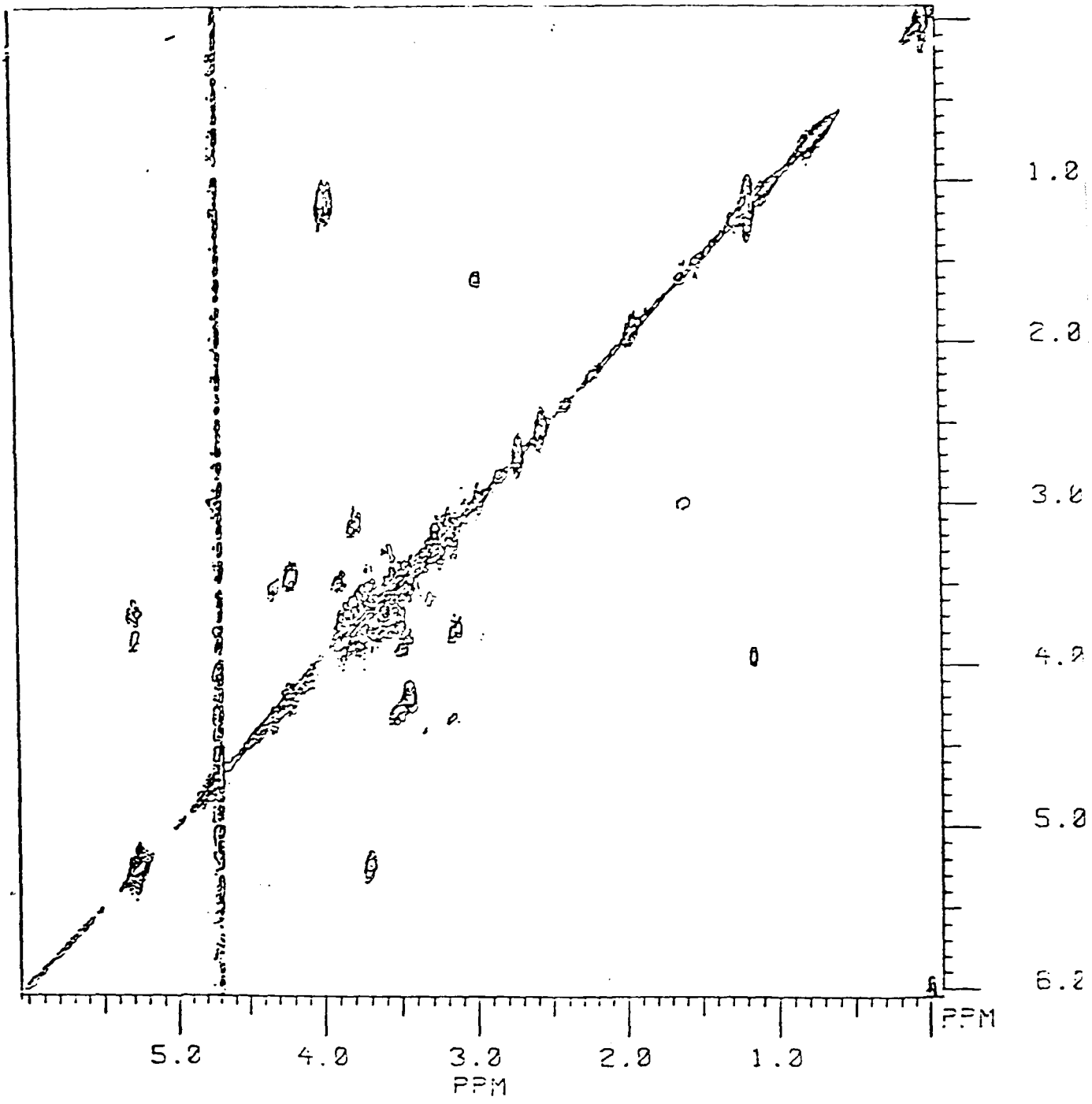


Figure 6 : A 400 MHz ^{1}H NMR spectrum of purified LPG in D_2O. DSS was used as internal reference. The spectrum was recorded at 22°C.

Figure 7: Counter plot of COSY spectrum of LPG in D_2O at 22°C. The spectrum represented in the unsymmetrized mode. On the tope is the one dimensional spectrum of LPG under similar conditions.




1.2
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5.0
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PPM
5.2
4.0
3.0
2.0
1.2
PPM

the migration of LPG was enhanced on thin layer chromatography by increasing pyridine. It is suggested that the amphipathic nature of the LPG is due to the presence of covalently bound lipid, and that the lipid served to anchor the glycoconjugate onto the parasite membrane. The LPG molecule behaved like phospholipids, when solubilized in water. It produced a micellar structure shielding the hydrophobic portion from water, and oriented itself in a highly branched carbohydrate chain out wards (Handman *et al.*, 1984). Previous studies have shown that migration of glycoconjugate on TLC plate is reduced in presence of high proportion of water in solvent system and usually a small amount of Triton X-100 (Turco *et al.*, 1984).

LPG is eluted from Sephadex G-200 column in the presence of NH_4OH containing EDTA solution. It was observed that NH_4OH as eluting solvent was useful as it avoids the precipitation of the LPG on the top of the column. The addition of EDTA in the eluting buffer was essential for preventing any aggregation of LPG. The aggregate of LPG molecule eluted in void volume of the column. King *et al.* (1987) suggested that LPG released into the culture medium bound tightly to serum albumin and eluted in the void volume following chromatography on Sephadex G-100, and the LPG-albumin complex could not be separated by separate treatment with 0.1 M EDTA, 2% deoxycholate and 6 M urea. A single broad peak of the LPG was obtained from column indicating that there is a heterogenous population of LPG molecule in the sample. LPG purified by conventional methods failed to remove LPG-associated protein contaminants as they were tightly bound to LPG (Moll *et al.*, 1989; Jardim *et al.*, 1991). These associated proteins were removed by the treatment with proteinase K enzyme and protein/peptide free LPG is obtained after purification from octyl Sepharose column (Ilg *et al.*, 1992).

From 25 ml of packed cells about 150.0 mg of crude LPG was obtained. This on partial purification yielded 31 mg of partially purified LPG and on final purification on octyl Sepharose column gave a final yield of about 15.0 mg of pure LPG. This yield of LPG from non pathogenic strain was found to be less as compared to that from pathogenic strain of *L. donovani* promastigotes (King *et al.*, 1987; Orlandi and Turco, 1987). This observation is in concurence to the earlier report where the yield of LPG from pathogenic strain of *L. donovani* and *L. major* promastigotes is high. Furthermore, in a recent study with *L. major* Kimsey *et al.* (1993) have shown that LPG obtained from a virulent (Vir 5) strain of parasite expressed larger LPG as compared to the corresponding LPG from an avirulent strain. This observation suggested that virulent strain parasites produced more LPG than the avirulent strain. The low expression of LPG in the avirulent parasite might be either due to a defect in LPG biosynthesis or due to its immaturation during metacyclogenesis of the parasite. Hence, it is also possible that non-pathogenic strain (UR6) may contain a lesser copy number of LPG molecules/cell as compared

to the pathogenic strain. Biochemical properties of the LPG of pathogenic and non- pathogenic strain have been summarized in table 1. This table indicates that both LPG's are different in reference to yield, presence of copy number/cell and structure but quite similar in molecular weight and mobility on TLC.

The purity of LPG was characterized by TLC. A single fused spot was achieved, suggesting that a single population of LPG molecule is present. The R_f value of the spot was found to be similar to that of the pathogenic strain (Orlandi and Turco, 1987) under similar conditions. It was observed that the LPG of a non-pathogenic strain (UR6) of *L. donovani* promastigotes have greater movement in hydrophobic solvent as compared to hydrophilic solution. It is suggested that UR6 LPG has more hydrophobic properties. Protein contaminants which were present in crude partially purified LPG, were removed by treatment with proteinase K enzyme. therefore, no positive silver stain band was observed on SDS-PAGE, suggesting that proteinase K treatment of LPG devoids it of any protein contaminants. It was supported by the NMR spectroscopy that no any $-NH_2$ signal was found during nMR studies except carbohydrates corresponding peaks were observed. Therefore, it was confirmed by ^1H NMR spectroscopic studies, that the LPG molecule in the sample was pure.

TABLE 1:

COMPARATIVE PHYSIO-CHEMICAL PROPERTIES OF LPG OF *L. donovani* PROMASTIGOTES

Properties	*Nonpathogenic strain*	*Pathogenic strain*[a]
Molecular Weight	20-25 kDa	15-30 kDa
Mobility on TLC	0.66±0.02	0.62
Yield	Low	High
Structure	Branched and more complex	Unbranched and comparatively simple
Cellular copy number		$1.5\text{-}3\times10^6$ molecule of LPG cell

a: Literature reports

CHAPTER 5

EFFICACY OF LIPOHOSPHOGLYCAN AGAINST *L. DONOVANI* INFECTION: *STUDIES IN MACROPHAGE SYSTEM*

INTRODUCTION

Leishmania promastigotes are introduced into the host blood circulation during a successful bite by the insect vector, the sandfly. They are then recognised and consequently internalized by the host macrophages to complete the biological cycle. Recognition and internalization into the macrophage is therefore, very critical feature in *Leishmania* cell cycle. The recognition of parasite by macrophage involves a receptor mediated mechanism. It is suggested that, both recognition and internalization of *Leishmania* parasite into macrophage is mediated by carbohydrate containing surface molecules (da Silva *et al.*, 1989; Puentes *et al.*, 1988; Talamas-Rohana *et al.*, 1990; Kelleher *et al.*, 1992; 1995). *In vitro* studies suggest that promastigotes may utilize several different macrophage receptors depending on the parasite species and developmental stage, and on whether serum is present. In the absence of serum, there is evidence that the two major macromolecules on the promastigotes surface, a complex carbohydrate lipophosphoglycan (LPG) and a glycosyl-phosphatidylinositol (GPI) anchored 63-kDa proteinase, gp63, are able to bind directly to a number of lectin like receptors of the macrophage (Kelleher *et al.*, 1992; Talamas-Rohana *et al.*, 1990; Wilson and Pearson, 1986; 1988). In the presence of serum, however, the promastigote surface is opsonized with complement components such as C3b and iC3b, and promastigote uptake occurs via the complement receptors CRI or CR3 (Mosser *et al.*, 1992 da Silva *et al.*, 1989).

It has been demonstrated that manose-fucose receptor (MFR) or mannan plays an important role in the attachment and internalization of *L. donovani* promastigotes to human monocyte derived macrophages (Wilson and Pearson, 1986). Two of the glycoconjugate, fucose-mannose glycoprotein ligand (FML) and phosphate mannogalactan ligand (PMGL) purified from promastigotes were found to be potent inhibitors of promastigotes phagocytosis (Palatnik *et al.*, 1989). As reported erlier, the attachment and internalization of *Leishmania* parasites is inhibited by low temperature, cytochalasins and mild fixation of macrophage with formalin, suggesting that parasite uptake is dependent on the integrity of the macrophage phagocytic mechanisms (Chang, 1979). It has been shown that the promastigotes derived LPG epitopes are identified only by parasite infected macrophages, when incubated at 37°C. None of the other cell types expressed LPG epitopes after incubation with live promastigotes. However, all of the cells including macrophages are strongly bound to LPG or PG at either 37°C or 4°C (Tolson *et al.*, 1990). It has been shown that disaccharide repeats of LPG of *L. donovani* accumulate on the surface of infected macrophages during parasite internalization. The LPG epitope is recognized by the monoclonal antibody CA7AE, found to be a phosphorylated Galβ1-->4Man α 1 disaccharide unit (Tolson *et al.*, 1989). Epitope interaction to infected macrophage is not dependent on lipid-tail interactions with the bilayer of the macrophage plasma membrane.

Although it may occur via interaction between carbohydrate portion of the LPG molecule and host cell receptors such as, the CD18 family of the integrins that are present on many cells including macrophages (Tolson *et al.*, 1989). LPG epitope could also be detected on the cell surface of amastigotes or its LPG and the infected macrophage using a number of monoclonal antibodies directed against several distinct determinants on the phosphoglycan moiety. Furthermore, the LPG expressed by the infected macrophage may be modified because unlike the parasite LPG which is expressed on promastigotes and amastigotes, it could not be radio labelled by galactose oxidase or periodate treatment.

Two functionally important LPG-domains involved in the biology of the parasite have been identified in *L. major* promastigotes. The repeat unit P3, PO_4-6[Gal(β1-->3)]Gal(β1-->3)]Gal(β1--->4)Man α 1-, has been shown to be involved in the attachment of procyclic promastigotes to the midgut epithelium of the sandfly vector, *P. papatasi* (Pimenta *et al.*, 1992), while the repeating unit P5b, PO_4-6[Gal(β1-->3) Gal(β1->3)Gal(β1-->3)]Gal(β1-->4)Man α 1- , is shown to be involved in attachement of infective *L. major* promastigotes to macrophage cell line J774 (Kelleher *et al.*, 1992). The interaction of P5b with macrophages appears to be *L. major* specific, suggesting that other *Leishmania* may use different domains of LPG or different surface molecules for host recognition. The epitopes on P3 and P4a recognized by the four MAbs which are expressed in LPG from both procyclic and metacyclic promastigotes. However, expression is low in amastigotes, as expected from the structure of amastigotes LPG (Kelleher *et al.*, 1994). P3 and P4a are also expressed at different relative abundance in the LPG of procyclic and metacyclic promastigotes. P3 decreases from 52 mol% in procyclic promastigotes to 31 mol% in the metacyclic form. while P4a increases from 9 to 45 mol%, respectively. However, the combined amount of P3 and P4a increases only slightly from 61 to 76 mol % in procyclic and metacyclic promastigotes, respectively. The intact LPG is a more effective inhibitor (30-100 fold) of 4A2-A2 and 2G11-A3 than P3 and P4a (Kelleher *et al.*, 1994). A much higher affinity of antibodies for intact LPG has also been shown for MAbs WIC 79.3 (Kelleher *et al.*, 1992), CA7AE and BF9CC (Tolson *et al.*, 1989). The MAb WIC 108.3, which recognizes the unsubstituted repeat unit P2, PO_4-6 Gal (β1-->4)Man α 1- , revealed a higher expression in the amastigotes than in the promastigotes. This is due to amastigote LPG containing 70 mol% of P2, while procyclic and metacyclic promastigotes contain only 7 and 15 mol% of P2, respectively (Kelleher *et al.*, 1994). Recently, the P3 repeat unit is shown to be involved in the attachement of procyclic promastigote to the epithelial cells of midgut (Pimenta *et al.*, 1992), while P3 is present in both procylic and metacyclic LPG's, only procyclic LPG could inhibit binding of parasite to epithelial midgut. This is thought to be accounted for by procyclic LPG having clusters of repeat units with side chains of β-Gal units. Metacyclic LPG on the

otherhand has a higher number of repeat units terminating with βAra (P4a and P5a), reducing the likelihood of clusters of repeat units with terminal β Gal residues. Hence, P3 in procyclic LPG is an attachment ligand for epithelial cells of the sand fly midgut (Kelleher *et al.*, 1994).

A recent report shows that LPG is capable of blocking the attachment of amastigotes or promastigotes to macrophages and also inhibit internalization of *L. major* promastigotes by macrophage (Kelleher *et al.*, 1995). This inhibitory effect has been shown to be mediated by β (1-->3) Gal residues specific to *L. major*. The amstigotes LPG also binds to primary macrophages and cell line. The binding of amastigotes to macrophages could be blocked by intact LPG from *L. major* amastigotes as well as LPG from promastigotes to several other *Leishmania* species, suggesting involvement of a conserved binding domain and not a species-specific domain (Kelleher *et al.*, 1995).

The protective potential of *L. donovani* LPG, isolated from promastigotes of *L. donovani* (strain UR6), against *L. donovani* infection in peritoneal macrophages has been studied. The studies demonstrate that UR6 LPG inhibits binding of promastigotes of Dd8 strain to peritoneal macrophages in a concentration dependent manner. Furthermore, UR6 LPG provided a significant protection (about 85-90%) in peritoneal macrophages against promastigotes infection.

MATERIALS AND METHODS:

Mouse Peritoneal macrophage culture:

Three month old BALB/c mice, in bred at the animal house facility at Central Drug Research Institute, Lucknow, India, were used to obtain peritoneal macrophages. 0.5 ml of 2% thioglycolate was injected intraperitoneally to each animals. They were sacrificed by overdose of ether or chloroform after 48 hr of thioglycolate injection. Animals were washed with 70% ethanol and the epidermis was cut exposing the dermis. Five milliliters of cold RPMI-1640 medium containing 100 µ/ml of penicillin and 100 µg/ml of streptomycin was injected into the peritoneal cavity. The abdomen was massaged gently and the peritoneal fluid was aspirated under sterile conditions, and stored on ice. The peritoneal fluid was centrifuged at 1000xg for 30 min at 4°C. The supernatant was discarded and the pellet was resuspended in warm medium, washed two times by centrifugation at 1000xg for 20 min at 4°C. The pellet cells were resuspended in 5.0 ml of complete RPMI-1640 medium containing 10% FCS (v/v) and 40 µg/ml gentamycin. A small drop of cell suspension was transferred on haemocytometer and counted under 1000x magnification. Complete medium was added to required amount for adjusting cells count $1x10^6$ cells/ml. The viability of the cells were checked by trypan blue staining.

Circular glass cover slips were cleaned with sulfochromic acid, extensively washed

in triple distilled water, sterilized by 70% ethanol then flame and placed in 24- well tissue culture costar trays (Corning, New York). In each well 1.0 ml of cell suspension was plated on to the cover slip and to it was added 1.0 ml of complete medium. The cells ($0.5x10^6$/ml) were allowed to adhere at 37°C in an atmosphere of 5% CO_2 for 5 hr or more. Nonadherent cells were removed by rinsing the plates with complete RPMI-1640 medium twice. Approximately $2x10^5$ cells were obtained in each well. Adherent peritoneal cells were incubated for an additional 18 hr under the same conditions and used for studies.

Trypan blue staining:

50 µl of trypan blue (0.01%) in PBS was added to the cell suspension and placed in ice. Trypan blue stains dead cells (blue colour) only by entering through their ruptured plasma membrane, leaving the viable cells-intact.

Phagocytosis assay:

Monolayers of peritoneal macrophages were prepared on glass cover slips in a 24 well costar trays. Each cover slip was coated with 2×10^5 macrophages in each well containing 1.0 ml of RPMI-1640 medium, supplemented with 10% Fetal calf serum (FCS) and were incubated at 37°C in 5% CO_2 atmosphere. Living promastigotes of *L.donovani* strain Dd8 or UR6 obtained (from 4 and 3 days culture respectively), were added to macrophages after two washing in an appropriate concentrations (2,5,10 and 25 parasite per macrophage) and incubated for 18 hr at 37°C in at 5% CO_2 atmosphere.

Promastigotes of *L.donovani* strains UR6 or Dd8 were added to monolayers of peritoneal macrophages in a constant ration (10 parasites per macrophage) in each well. The tissue culture plates were incubated for periods ranging from 15 min, to 24 hr at 37°C in 5% CO_2 atmosphere in CO_2 incubator (Heraeus, Jouan, IG 150). Each well was washed after its incubation periods, three times with RPMI-1640 medium to remove free promastigotes. Cover slips were fixed in methanol for 10 min and stained with 10% Giemsa stain (E.Merck AG, Darmstadt, Federal Republic of Germany) for 30 min, dried, mounted, and analyzed microscopically. The phagocytic index was determined by counting the percentage of all macrophages in 10-12 microscopic fields under 1000x magnification (a total of approximately 500 cells) that had phagocytosed at least one *Leishmania* parasite and multiplying this count by the average number of *Leishmania* parasites per macrophage observed in 100 macrophages (Palatnik *et al.*, 1989). For determination of adhesive index, the experiment was carried out at 4°C keeping the other experimental conditions same as above (Bianco *et al.*,1975).

Infection of Macrophages:

Promastigotes harvested in the late log to stationary phase were used to infect culture of adherent macrophages on cover slips at a ratio of 5 parasites per macrophage. Infection was performed at 37°C in 5% CO_2 atmosphere. The percent infected cells and the number of amastigotes in these cells were counted at specific intervals by removal of all nonadherent or extracellular promastigotes by washing with PBS.

Liposomes Preparation for *in vitro* studies:

Lipid film was prepared as mentioned in Chapter VI. Dried film was dissolved in RPMI-1640 medium without FCS and LPG at required concentration (10, 50, 100, 1000 and 2000 ng/well) was added to it. All preparations were vortexed and sonicated in the presence of ice, pulse cycle of 50/sec. and 3 Hz frequency was maintained for 15-20 min with a time interval of 2 min. It was centrifuged at 30,000 x g for 15 min. Supernatant was collected in the fresh tubes. All preparations of LPG or liposomised LPG were filtered with 0.2 μm membrane filter (Sartorious) in the sterilized (autoclaved) tubes and made upto 3 ml in each tube by adding complete medium, i.e., FCS supplemented medium. The same phospholipids to cholesterol ratio was maintained for all experiments.

Macrophage binding assay:

Monolayers of peritoneal macrophages ($2x10^5$ cells/ml) were grown on cover slips as described above. These macrophages were incubated with LPG alone and with liposomised LPG (LPG in SUVch) for 2 hr, prior to the addition of parasites to the macrophages. Promastigotes of strain Dd8 were added to macrophage monolayers at ratio of 5 parasites per macrophage and incubated for 45 min at 37°C in 5% CO_2 atmosphere. The binding assay was terminated by three gentle washing with PBS, for removal of free promastigotes. The cover slips were fixed in methanol, stained with 10% Giemsa for 30 min, dried, mounted and examined microscopically by counting number of macrophages with bound promastigotes. A minimum of 500 macrophages were counted from each cover slip.

Inhibition of *Leishmania* internalization in macrophages by LPG:

Monolayers of peritoneal macrophages ($2x10^5$ cells/ml) were grown on cover slips and incubated with LPG or liposomised LPG (LPG in SUVch) of different concentration for 2 hr prior to the addition of parasites to the macrophages. Promastigotes of strain Dd8 obtained from 4 days old culture were added to macrophage monolayers at a ratio of 5 parasites per macrophage and incubated for 6,12,18 and 24 hr at 37°C in 5% CO_2 atmosphere. Extracellular

promastigotes were removed by washing with RPMI-1640 medium after 6 hr.

After incubation period, experiment was terminated by three subsequent washes with PBS (0.1M, pH 7.2), for removal of free organisms. The cover slips were fixed in methanol for 10 min, stained with 10% Giemsa stain for 30 min, dried, mounted and examined microscopically . Number of the infected macrophages and amastigotes were counted for each cover slip.

Pre and Post treatment of LPG:

Monolayers of peritoneal macrophages ($2x10^5$ cells/ml) were grown on cover slips as described above. The cells were incubated with promastigotes (5 promastigotes/macrophage) for 6 hr at 37°C in 5% CO_2, 93% N_2 and 2% O_2 in a CO_2 incubator. Extracellular promastigotes were removed by thorough washing. LPG (100 ng/well) was added in each well and incubated for 2 hr in the same environments. After washing the plate was incubated for 18 hr in CO_2 incubator.

In another set of experiment, the macrophage monolayer was incubated with LPG (100 ng/well) for 2 hr, prior to the challenge with promastigotes for 6 hr in 5% CO_2 atmosphere. Extracellular promastigotes were removed. Fresh complete medium was added and incubated for 18 hr as described above. Controls were prepared in the absence of LPG.

RESULTS:

Infectivity of promastigotes of Dd8 strain in peritoneal macrophages:

The macrophage culture system was used to evaluate the *in vitro* infectivity of *L. donovani* promastigotes (Dd8). The infectivity of *L. donovani* promastigotes of strain Dd8 was examined by incubating promastigotes in stationary phase of growth with mouse peritoneal macrophages. *L. donovani* promastigotes showed a high levels of infection as observed by the high count of amastigotes in the glass slides after 24 hrs of incubation period. Further increasing the time of incubation showed the number of amastigotes increased inside the infected macrophages and released of some amastigotes from the macrophages due to ruptured the plasma membrane.

Phagocytosis assay:

In vitro phagocytosis assay using promastigotes of strain Dd8 and UR6 with peritoneal macrophages obtained from BALB/c mice was carried out. The effect of incubation of promastigotes of Dd8 and UR6 strains of *L. donovani* with peritoneal macrophages under

similar conditions showed that the infectivity of Dd8 promastigotes was more compared to UR6 promastigotes (Plate 1).

The effect of promastigotes/macrophage ratio on phagocytosis was also studied. The phagocytic index (PI) as shown in figure 2A was found to be proportional upto a ratio of 10:1 with respect to promastigotes macrophage ratio. Increasing the promastigotes/macrophage ratio further upto 25:1, did not show any significant changes in phagocytic index. The adhesion contributed only 7% or less to the phagocytic index as observed by carrying out the assay at 4°C. Thus, the observed internalization of promastigotes was indicative of a normal physiological phenomenon.

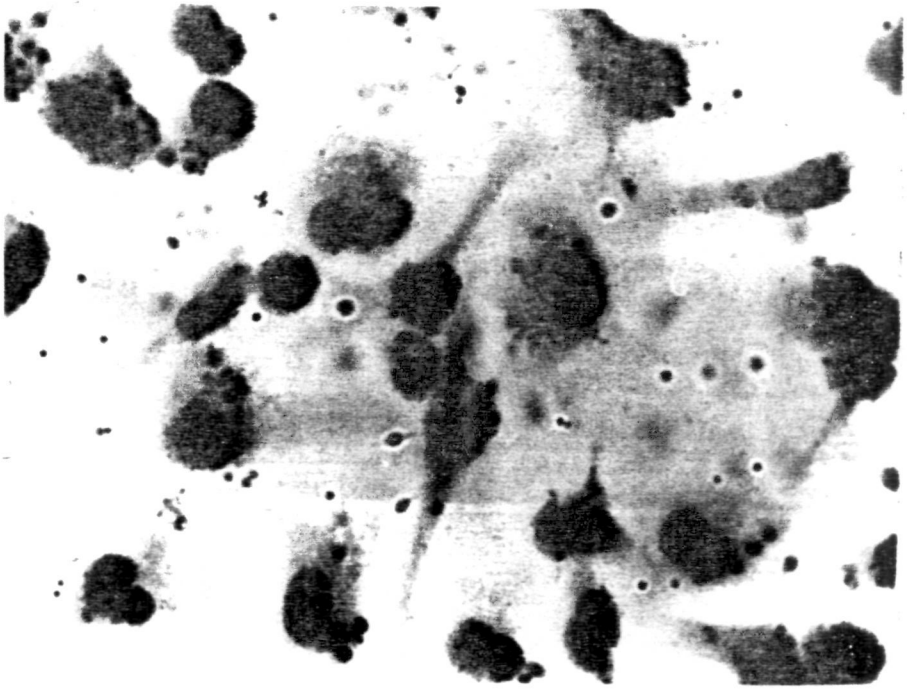
Time dependent phagocytosis of promastigotes by macrophages was also studied. Figure 2B shows the time dependent internalization of promastigotes of strains Dd8 and UR6. The phagocytic index was found to be directly proportional to the incubation period upto 6 hrs, when maximum number of promastigotes per macrophage were internalized and their phagocytic capacity seemed to be saturated. The examination of infected macrophage culture after 18 hrs post challenge with promastigotes should a large number of amastigotes in the infected cells, for both strains. The percentage of infected peritoneal macrophages with Dd8 promastigotes was found to be higher as compared to UR6 promastigotes. These observations suggest that promastigotes of non-pathogenic UR6 strain of *L. donovani* are able to bind, infect and proliferate in the peritoneal macrophages.

LPG blocks attachmentof promastigotes to peritoneal macrophages :

Studies were carried out to evaluate the potential of UR6 LPG in inhibiting the attachment of Dd8 promastigotes to peritoneal macrophages. With increasing ratio of parasite to macrophage, a concomitant increase in the percentage of cells with attached promastigotes or number of promastigotes attached to each cell was observed. At the ratio of 5 promastigotes per macrophages, with an incubation time of 45 min, only one or two parasites were found to be attached to each cell.

The effect of pretreatment of peritoneal macrophages with varing concentrations of UR6 LPG on the macrophage promastigote interaction is shown in figure 3. A concentration dependent increase in LPG's ability to inhibit binding of Dd8 promastigotes to peritoneal macrophages. For 10 ng/ml LPG about 50% inhibition of attachment to peritoneal macrophages was observed. This inhibitory activity was enhanced from 60% to 78%, when the LPG concentration was increased from 50 ng/ml to 100 ng/ml, respectively. A maximum inhibition of about 85-90% was observed for 1000 ng/ml of LPG. No further enhancement in inhibition with increase in LPG concentration to 2000 ng/ml was observed. Similar results were obtained,

Figure 1: Infectivity of promastigotes of *L. donovani* strains UR6 and Dd8 strains in peritone macrophages.



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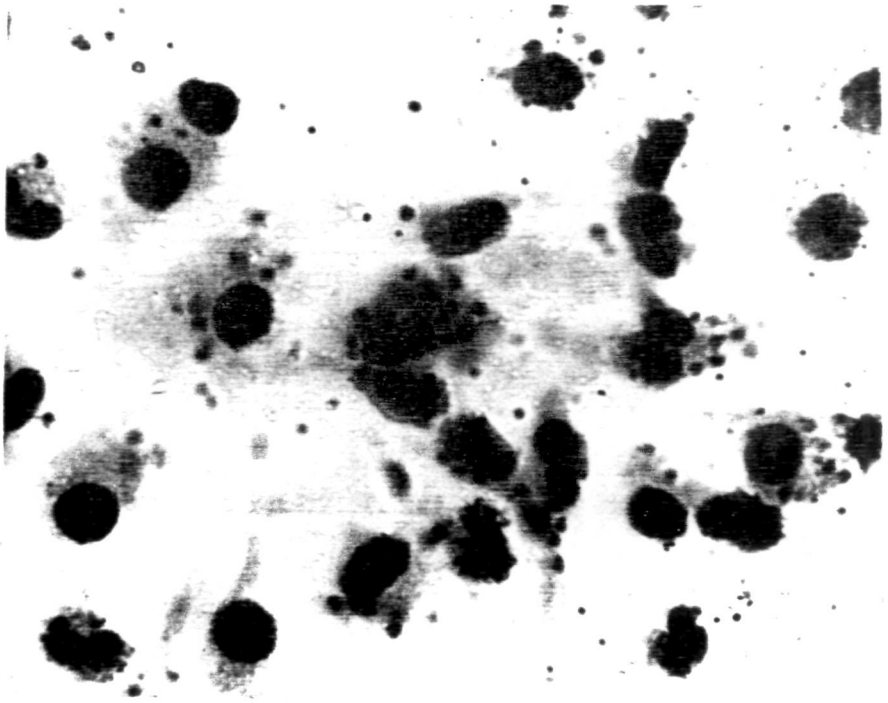

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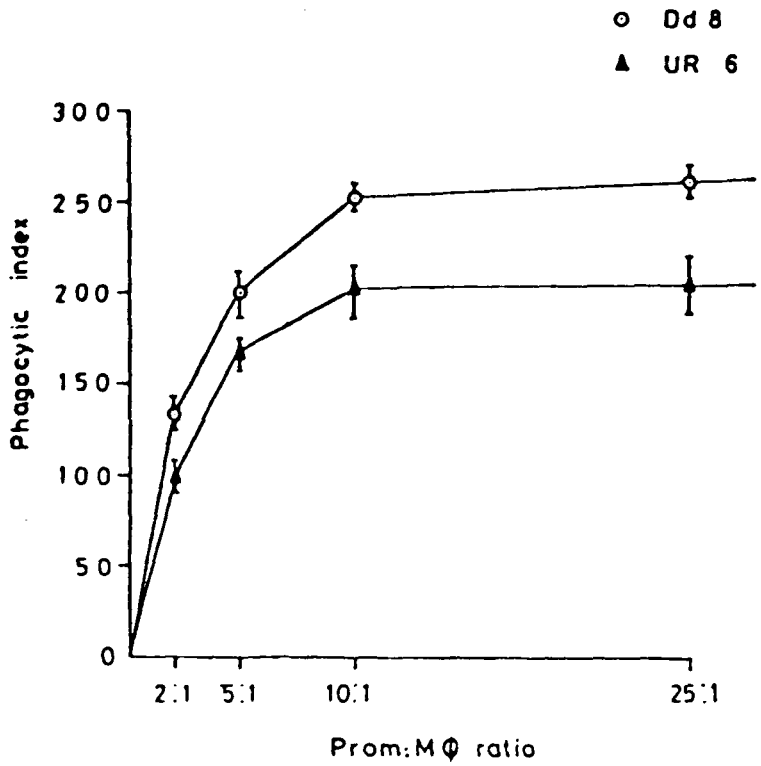

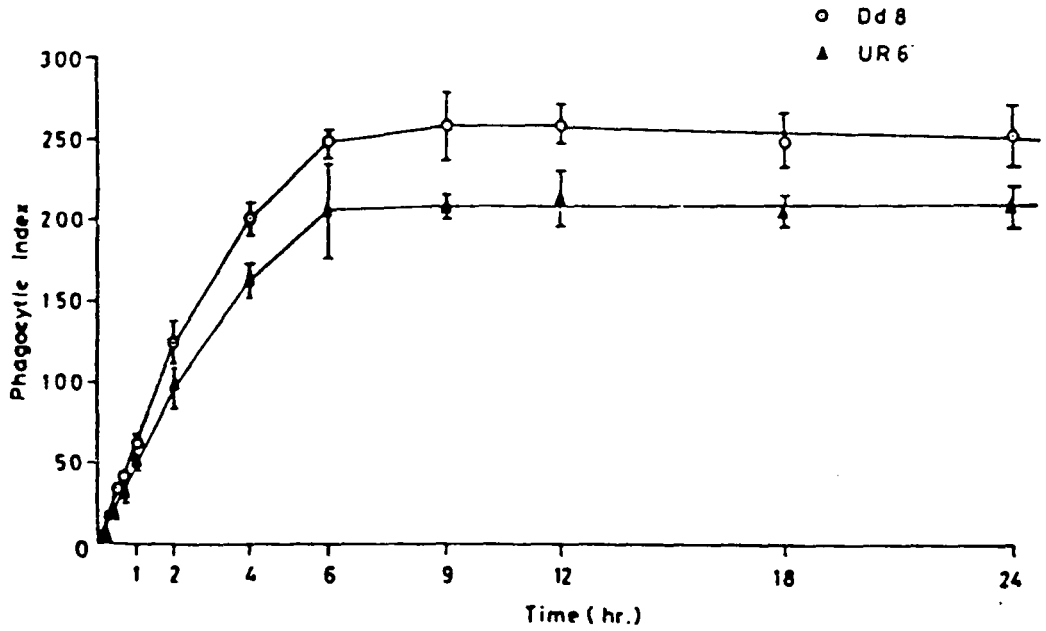

Figure 2: Effect of promastigote/macrophage ratio (6 h incubation) (a) and effect of time at a ratio of 5:1 promastigote/macrophage (b) on phagocytosis.
Values are mean ± SD for three experiments.

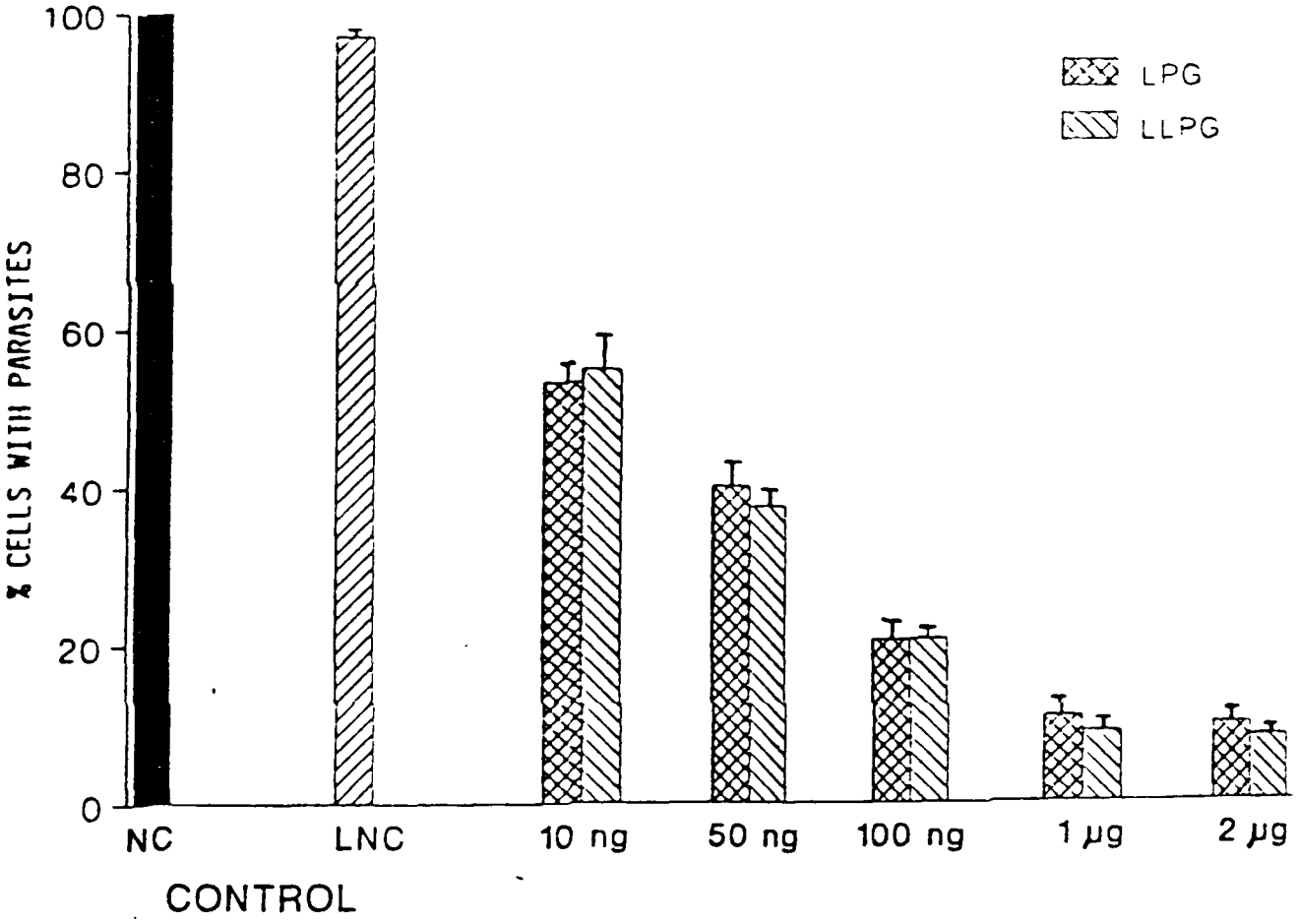

Figure 3: Inhibition of attachment of *L. donovani* promastigotes to mouse peritoneal macrophages in presence of UR6-LPG. The number of macrophages with parasite attached was counted and expressed as percent adherence compared to control. In the figure the abbreviation L-LPG stands for LPG incorporated in cholesterol containing small unilameller vesicle. Values are mean ± SD for four experiments testing each condition in triplicate.

when the LPG was incorporated into small unilamellar vesicles (SUV) with cholesterol. These results demonstrate that UR6 LPG inhibits attachment of promastigotes of Dd8 strain to peritoneal macrophages in a concentration dependent manner.

Protection of peritoneal macrophages against *Leishmania* infection using UR6 LPG

Peritoneal macrophages were incubated with UR6 LPG for 2 hrs followed by challenge with *L. donovani* (Dd8) promastigotes. The intensity of infection in macrophages was assayed by counting the percent cells with amastigotes, and the results shown in (Figure 4 and summarized in Table 1). A dose dependent protection in macrophages against *Leishmania* infection was observed when macrophages were preincubated with LPG. A subsequent decrease in infected cells with increasing concentration of LPG or liposomised LPG molecule was observed (Table 1). A maximum protection of about 85 percent was observed for 1000 ng/ml of LPG. Incorporation of LPG in liposomes did not show any substantial difference as compared to LPG alone treatment.

Pre and Post Treatment:

In order to look into the possibility whether LPG mediated protection in macrophages against *L. donovani* was either due to inhibition of attachment of promastigotes to macrophages or killing of amastigotes inside the macrophages, studies with pre and post treatment of macrophages with UR6 LPG were carried out. Peritoneal macrophages were either challenged with Dd8 promastigotes for 6 hrs followed by the removal of free promastigotes by washing with PBS and incubating with LPG for 2 hrs or incubating macrophages with LPG for 2 hrs followed by the removal of unbound LPG and challenging with Dd8 promastigotes for 6 hrs. Table-2 shows the intensity of infection in different groups of animals at 18 hrs post infection. Figure 5 also shows protective efficacy of LPG molecule against *L. donovani* infection. Pretreatment of macrophages with LPG showed a substantial inhibition of infection but no inhibitory effect on infection was observed when macrophages were incubated with LPG post establishment of infection. These observation suggests that LPG dependent inhibition of attachment of promastigotes to macrophages is the primary mechanism responsible for LPG mediated protection of peritoneal macrophages against *L. donovani* infection.

Thus, above presented results demonstrate that LPG of *L. donovani* promastigotes (strain UR6) inhibit the attachment of promastigotes of pathogenic strain (Dd8) to the peritoneal macrophages. Furthermore, preincubation of peritoneal macrophages with LPG provided significant protection against *L. donovani* infection.

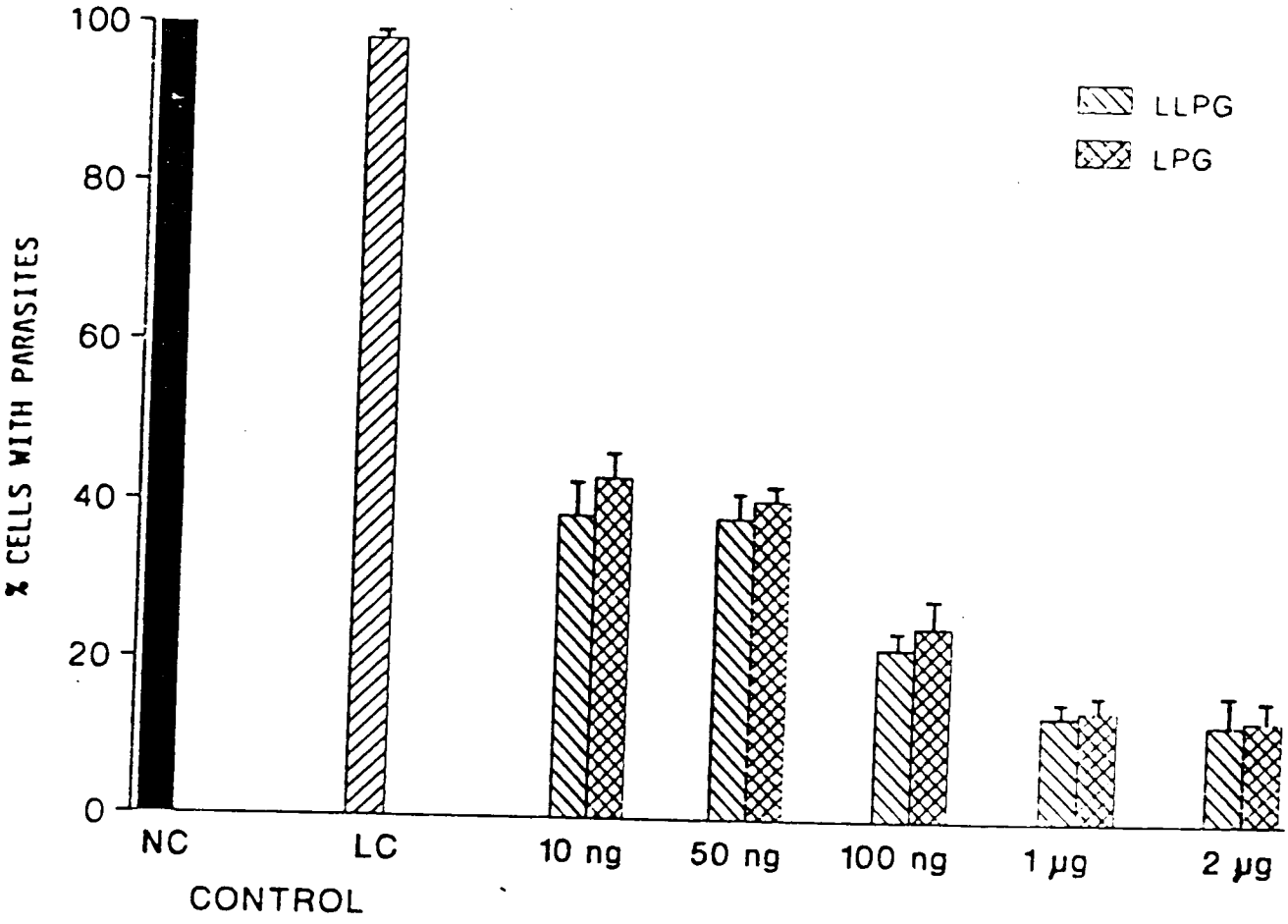

Figure 4: Effect of pretreatment with UR6-LPG on intensity of *L.donovani* promastigotes infection (strain Dd8) in BALB/c mice peritoneal macrophages upto 18 h post infection. The number of macrophages with parasite internalized was counted. In the figure the abbreviation L-LPG stands for LPG incorporated in cholesterol containing small unilamellar vesicle. Values are mean ± SD for four experiments testing each condition in triplicate.

Table 1:

Development of *L. donovani* promastigote infection in peritoneal macrophages in presence and absence of pretreatment with UR6 LPG.

Treatment Groups	% infected cells after		No. of parasites/100 cells	
	6 h	18 h	6 h	18 h
1. Control	69±2.02	80±3.1	191±5	239±18
2. 10ng LPG	35±2.16	36±1.2	44±1.7	74±5.03
3. 50ng LPG	27±1.13	32±2.01	33±1.3	56±3.08
4. 100ng LPG	22±0.9	23±1.03	33±0.7	45±2.5
5. 1µg LPG	11.9±0.3	11.5±0.4	27±1.2	32±0.4
6. 2 µg LPG	10.6±0.4	11.1±0.2	25±1.2	28±0.6
7. 10ng LLPG	30±2.04	31±1.2	44±1.82	56±2
8. 50ng LLPG	26±0.2	30±0.12	36±2.1	49±1.32
9. 100ng LLPG	18±1.01	19±1.09	36±1.9	32±1.5
10. 1µg LLPG	11±1	10.8±0.3	19±1.2	30.7±0.9
11. 2µg LLPG	10.8±0.4	10.2±0.2	17±0.62	28±0.7

Values represent mean ±SD for four experiments testing each condition in triplicate.

Peritoneal macrophages were incubated with UR6 LPG for 2 h followed by removal of free LPG l washing as described in materials and methods. They were then challenged with *L. donovani* promastigot (strain Dd8) at a ratio of 1:5. The infectivity of promastigotes was checked at 6 and 18 h post infectic In the table the abbreviation LLPG stands for LPG incorporated in cholesterol containing unilamell liposomes.

TABLE 2:

Protection against *L. donovani* infection in BALB/c mice peritoneal macrophages on pre and post treatment with UR6 LPG.

Treatment	% Infected cells	Amastigotes/100 cells
1. Control	76±2.5	226 ± 49
2[a]. Post treatment	75 ± 2.9	215 ± 5.3
3[b]. Pre treatment	24.5 ±0.7	59 ± 7.3*

Values represent mean±SD for three experiments testing each condition in duplicate.

a: Peritoneal macrophages were incubated with promastigotes of strain Dd8 for 6 h followed by incubation with LPG (100 ng/well). The data was collected 18 h post infection.

b: Peritoneal macrophages were incubated with LPG for 2 h followed by incubation with promastigotes of strain Dd8 for 6 hrs. The data was collected 18 h post infection.

* p value as determined by student t test $p < 0.001$

Figure 5: Protection against *L. donovani* infection in BALB/c mice peritoneal macrophages on pre and post treatment with UR6 LPG:(a) untreated peritoneal macrophage challenged with promastigotes (control) (b) peritoneal macrophages were incubated with promastigotes of strain Dd8 for 6 h followed by incubation with LPG (100 ng/ml) c peritoneal macrophages were incubated with LPG for 2 h followed by incubation with promastigotes of strain Dd8 for 6 hrs. The data was collected 18 h post infection.


a
b
c

DISCUSSION:

LPG is an attachment ligand for promastigotes which is involved in a direct interaction with macrophage receptors (Talamas-Rohana *et al.*, 1990) or it can be opsonized with complement components to interact with macrophage receptors (da Silva *et al.*, 1989). LPG from all the species of *Leishmania* examined share the same tripartite structure comprising of a backbone of disaccharide repeat units, which are linked by a phosphosaccharide core to a lyso-alkyl phosphatidylinositol anchor (Turco and Descoteaux, 1992). Recent studies have demonstrated that binding of amastigotes to macrophages could be blocked by intact LPG from *L. major* amastigotes, as well as by LPG's from promastigotes of *L. major*, *L. donovani* and *L. mexicana* (Kelleher *et al.*, 1995) indicating the possibility of involvement of conserved non species-specific LPG domain in LPG-macrophage binding. In the presence of complement, the attachment of amastigotes to macrophages is not altered, suggesting the LPG interacts directly with macrophage receptors (Kelleher *et al.*, 1995). It has been demonstrated that the glycan core of LPG can reduce the attachment of amastigotes to macrophages (Kelleher *et al.*, 1995). This inhibition has been shown to be dose dependent and decreases with increasing ratios of amastigotes to cells. (Kelleher *et al.*, 1995).

Studies were carried out to evaluate the efficacy of heterologous LPG in providing protection against *L. donovani* infection. The reason for choosing LPG of promastigotes of strain UR6 for these studies was that, they can be grown in large quantities in the semisolid medium as described in the materials and methods. Hence, large amount of pure LPG can be obtained easily from promastigotes.

The interaction between *Leishmania* promastigotes and BALB/c mice peritoneal macrophages was studied *in vitro*. The effect of infectivity of both strains of *L. donovani* promastigotes (Dd8 and UR6) was checked. It was found that the Dd8 promastigotes had greater infectivity than UR6 strain. The effect of the promastigote macrophage ratio on phagocytosis was also studied. The rate of phagocytosis increased upto 10 promastigotes per macrophage and beyond that the phagocytic capacity of the macrophages seemed to be saturated. The internalization of promastigotes by the macrophages was a physiological phenomenon representing a normal phagocytic macrophage activity. The morphological changes in internalization of promastigotes during the course of study was not observed and the plateau was not an equilibrium between internalization and digestion of parasites in macrophages.

The effect of time on promastigotes internalization by peritoneal macrophages was assessed. The phagocytic index was found to be proportional to the incubation period upto 6 hrs, where the maximum number of promastigotes per macrophage were internalized. After 6 hrs, approximately 80-85 percent of macrophages were capable of internalizing the promastigotes,

and after which their capacity seemed to be saturated. Therefore, the phagocytic index indicates that the maximum internalization occurs upto 6 hrs. Hence, it could be suggested that the ratio of 5-10 promastigotes per macrophage and an incubation time of 6 hrs is the suitable condition for *in vitro* assay.

Previous studies have shown that promastigote LPG from *L. major* inhibited *L. major* promastigote binding to macrophage cell line J774 (Kelleher *et al.*, 1992) and LPG from *L. major* amastigote is also inhibited the attachment of amastigote to the macrophage cell line J774 (Kelleher *et al*, 1995). The potential of UR6 LPG in providing protection against *L. donovani* infection was also studied. Purified LPG from *L. donovani* promastigotes (strain UR6) was able to inhibit the attachment of promastigotes of *L. donovani* strain Dd8 to peritoneal macrophages. It was evident from the studies conducted, that inhibition of attachment of promastigotes to peritoneal macrophage was concentration dependent. These results support the earlier observation (Kelleher *et al.*, 1995) that domain of LPG which is involved in binding of promastigotes to the macrophages, is conserved in the promastigotes LPG of both the Dd8 and UR6 strains of *L. donovani*.

Two mechanisms of binding of the glycoconjugate to cells may be postulated: a specific mechanism, by which the carbohydrate part of the molecule binds to macrophages, and a non-specific mechanism by which a hydrophobic (probably lipid) part of the molecule binds to all cells. It is therefore, interesting to note that the glycoconjugate bound to macrophages (presumably via a specific receptor) was capable of capping, while the glycoconjugate, which was merely inserted into the membrane of other cells by hydrophobic interaction, was not (Handman and Goding, 1985). It has been suggested that *L. major* glycoconjugate is the parasite molecule involved in macrophage recognition and attachment, and that this interaction occurs via the carbohydrate part of the molecule (Handman and Goding, 1985). In many other cell-cell interactions studied, the process of attachment has been demonstrated to be a multistep process. For example, attachment of leukocytes to endothelial cells may be mediated initially by a low-affinity binding interaction to the endothelial surface via selections and the Sialyl Lewis saccharide, followed by high affinity interaction with CD18 integrins of the endothelial cells providing stable association before transmigration (Lasky, 1992). Evidence also suggests that the attachment of *Plasmodium falciparum* merozoites to human erythrocytes is mediated by a two stage binding process utilizating two distinct binding domains of the 175-kDa erythrocyte binding antigen (Kain *et al.*, 1993). Similar interaction may also occur for the attachment of *Leishmania* amastigotes to macrophages via two binding domain of LPG or two different cell surface molecules (Kelleher *et al.*, 1995). The involvement of two cell surface molecules in a multistep infection process has already been demonstrated for attachment of *Leishmania* promastigote.

Beads coated with promastigotes gp63 were not internalized by macrophage, although attachment of these beads to the surface of the cells was observed (Russell and Wright, 1988). In contrast, beads coated with LPG attached and were phagocytised. Recently, Love *et al.* (1993) have also suggested that the interaction of *L. mexicana amazonensis* amastigotes with mammalian cells via interaction with heparin sulfate proteoglycans may be a general mechanism to bind to host cells. However, in the present study binding of promastigotes to peritoneal macrophages was inhibited by about 85-90% when pretreated with LPG, suggesting additional mechanisms for promastigote recognition by macrophages. This study is also supported by previous study (Kelleher *et al.*, 1995) that LPG was unable to completely block attachment suggesting the involvement of other ligands support the earlier observation (Kelleher *et al.*, 1995) that domain of LPG which is involved in binding of promastigotes to the macrophages, is conserved in the promastigotes LPG of both the Dd8 and UR6 strains of *L. donovani*.

After demonstrating the capability of UR6 LPG in inhibiting the attachment of Dd8 promastigotes to macrophages, it was necessary to see whether this can render any protection against *L. donovani* infection. It was observed that UR6 LPG is capable of providing significant protection (about 85 percent) in peritoneal macrophages against promastigotes upto 18 hrs post infection. The long term protection against *L. donovani* infection in peritoneal macrophages was also found to be concentration dependent. About 85 percent protection was obtained at 1000 ng/ml which failed to increase subsequently increasing the LPG concentration upto 2000 ng ml. This long term protection could have been obtained due to an inhibition of attachment of promastigotes to peritoneal macrophages or by killing of the amastigotes inside the macrophages. In order to destinguish between the peritoneal to processes macrophages were treated with LPG before and after challenge with promastigotes. It was observed that the peritoneal macrophages which were treated with LPG before being challenged with promastigotes provided significant protection against *L. donovani* infection. However, those which were challenged with promastigotes and then treated with LPG provided similar pattern of infection as observed that for control. These observations, suggest that pretreatment of peritoneal macrophages is necessary condition for achieving protection which is dependent on the inhibition of attachment of promastigotes to peritoneal macrophages. The protective efficacy was not enhance further by using LPG incorporated in SUV containing cholesterol. The interaction of peritoneal macrophages with promastigotes is receptor mediated. A number of studies focusing on the macrophage side of the interaction with *Leishmania* species showed that several host receptor may be involved (Wyler *et al.*, 1985; Blackwell *et al.*, 1985; Guy and Belosevic, 1993; Straus *et al.*, 1993). The LPG molecule of *Leishmania* parasite gets attached directly to the macrophage receptors and blocks these receptors resulting into inhibition of ingestion of promastigotes into macrophages.

CHAPTER 6

EFFICACY OF LIPOPHOSPHOGLYCAN AGAINST EXPERIMENTAL VISCERAL LEISHMANIASIS

INTRODUCTION:

Protozoan parasite of the genus *Leishmania* affects mammalian hosts, and causes a wide spectrum of diseases, depending on the host and on the *Leishmania* species involved. In humans, the infection ranges from self healing lesions to disseminated cutaneous disease or highly destructive mucosal lesions and from asymptomatic infection to fatal visceral dissemination, causing one of the World's major health problems.

Lipophosphoglycan (LPG) is one of the major *Leishmania* cell surface glycoconjugate and has been reported to exist on the surface of all *Leishmania* species of promastigote stage (Turco and Descoteaux, 1992) and also on the surface of *L. major* amastigotes (Glaser *et al.*, 1991, Moody *et al.*, 1993) and the flagellar pocket of *L. mexicana* (Bahr *et al.*, 1993). The abundance and presence of LPG thoughout the various stages of the life cycle of parasite suggest that it plays an important role in the biology and infectivity of *Leishmania* organisms. (Tolson *et al.*, 1990). Several studies have examined LPGs potential as a chemically defined vaccine against cutaneous leishmaniasis. Administration of immunoaffinity purified *L. major* LPG along with adjuvant *C. parvum*, to genetically resistant mice was found to induce full protection against challenge with promastigotes whereas, partial protection was observed in susceptible mice (Handman and Goding 1985; Handman and Mitchell, 1985; McConville *et al*, 1987). Similar protection against *L. mexicana* in CBA/Ca mice was also reported using homologous LPG reconstituted into liposomes (Russell and Alexander, 1988; Kahl *et al.*, 1990). However, it has been recently found that LPG purified by immunoaffinity is heavily contaminated with peptide. Mendonca *et al.* (1991) reported that T-lymphocytes from cutaneous leishmaniasis patients respond to purified *L. braziliensis* LPG, whereas proteinase K-treated LPG did not stimulate any response. Subsequent T-cell proliferation studies showed that the LPG-associated proteins were potent stimulators of T-cells in leishmaniasis patients (Moll *et al.*, 1989; Jardim *et al.*, 1991; Russo *et al.*, 1992) as well as in mice immunized with protein contaminated LPG (Jardim *et al.*, 1991). In view of these observations the mechanism of protection and use of LPG as a vaccine against leishmaniasis is open to question. Furthermore, use of LPG in protecting against visceral leishmaniasis has not yet been evaluated.

Studies on the potential of promastigote LPG in providing protection against *L. donovani* infection in susceptible golden hamsters was carried out. The results demonstrate that pretreatment of hamsters with LPG provides significant protection against *L. donovani* infection. Furthermore, the protective efficacy of LPG molecules was found to be significantly enhanced on its incorporation in cholesterol containing phosphatidylcholine liposomes.

MATERIALS AND METHODS:

Isolation and purification of egg phosphatidylcholine (PC):

The egg yolk was collected in grinder from one dozen egg and to this acetone (150-200 ml) was addded. This was mixed properly and filtered. Filtrate was discarded and solute was again treated with acetone. This step was repeated 9-10 times until filtrate became colourless or materials looked like white powder. It was dried in vacuo for 1.0 hr. The dried material was taken in round bottom flask and added 1.0-1.5 litre of absolute alchohol and kept on magnetic stirrer for 1-1.5 hr, filtered it and filtrate was evaporised at 40 - 45°C. The residue was dissolved in minimum amount of petrolium ether (b.p 60-80°C) and precipitated with chilled acetone. The solvent from precipitated material was removed by decantation. The precipitate was dissolved again in petrolium ether immediately to avoid oxidation of egg phosphocholine. Finally, it was precipitated with chilled acetone and all precipitate was dissolved in minimum amount of chloroform and rotavaporised, dried under vacuum and stored at -20°C. This dried material was passed through column chromatography over neutral alumina (Grade III) using increasing amounts of methanol in chloroform as the eluent. Elution with 7-10% methanol in chloroform gave pure egg PC. Fractions were analyzed by thin layer chromatography using silica gel G-60 TLC plates. The plates were developed in chloroform:methanol:water (65:25:4) and the spot was identified by staining them with iodine vapour followed by molybdenum-blue spary (Goswami and Frey, 1971).

Preparation of lipid liposomes:

Liposomes were prepared by the method of Gupta and Bali, 1981, using egg phosphocholine in presence or absence of cholesterol. Egg PC and cholesterol were taken in the round bottom flask and dissolved in minimum amount of chloroform. The solvent was evaporated in a rotavapor resulting in formation of a lipidfilm. For complete dryness, it was kept under vacuum for 1.0 hr. A ratio of 1 µg LPG to 1.0 mg phospholipid and 15% cholesterol of lipid was maintained in all experiments.

Neutral liposomes: Neutral liposomes were prepared using neutral phospholipid and cholesterol.

Multilamellar vesicles: Multilamellar vesicles (MLV) were prepared as described earlier (Senior and Gregoriadis, 1982; Woeff and Gregoriadis, 1984). Lipid film was prepared as mentioned above. The film was dissolved in 50 mM PBS (pH 7.4) vortexed and sonicated at 20°C for 30 min using water bath sonicator. The MLV was used immediately for immunization experiment.

Small Unilamellar vesicles: Small unilamellar vesicles (SUV) were prepared through probe

sonication of MLV in the presence of ice for 30 min or more, with 50 pulse cycle in 3 Hz frequency. Then preparations were centrifuged at 30,000xg for 15-20 min and collected supernatant. Again small unilamellar vesicles were washed with PB5 (50 mM, pH 7.4) by centrifugation at 30,000 x g. These preparations were used immediately for immunization, although storage at -20°C did not appear to alter their stability.

Negatively charged liposomes: Multilamellar and unilamellar vesicles were prepared as described above. 7% dicetylphosphate to phospholipid was added during the preparation of film. A ratio of 1 μg LPG to 1.0 mg phospholipid (egg PC) and 15% cholesterol of lipid was maintained in all preparations.

Positively charged liposomes: All preparations were prepared as mentioned above. Stearylamine (14%) of lipid (w/w) was added during the preparations of film. All preparations were used immediately for immunization.

Incorporation of LPG into liposomes:

LPG was incorporated into the liposomes by dissolving the film in 50 mM PBS (pH 7.4) followed by adding LPG stock as required amount before sonication. LPG incorporation was different in various liposomised preparations. Small unilamellar vesicles were incorporated more LPG as compared to multiplamellar vesicles. The percent of incorporation varied between 85-95%.

***IN VIVO* EFFICACY OF LIPOSOMISED LPG:**

For evaluating immunization / immunoprophylactic efficacy of liposomised LPG, various protocols were tried, which are summarized below.

Protocol-1:

Male syrian golden hamsters (35-45 gm) were given intraperitoneal injection of LPG alone (10 μg of LPG/animal) and in various liposome incorporated forms, like (SUV) and (MLV) liposomes. Each group was subdivided into two groups, which consisted of 5-7 animals/group. On day 21 post LPG administration, the hamsters were challenged with $1x10^7$ promastigotes of infective *L. donovani* strain Dd8. One set of subgroup was immunized again after 14 days (booster dose). The animals were sacrificed on day 45 and/or 60 post infection and the percent infected splenic macrophages were counted and percent protection was calculated.

$$\text{Percent protection (PP)} = \frac{N_1 - N_2}{N_1} \times 100$$

Where N_1 is the number of amastigotes/100 cells in control, N_2 is the number of amastigotes/100 cells in the treated group of animals.

Protocol-II:

Initially three groups were taken and subdivided into two groups. Each sub group contained 7 animals. Different concentration of LPG (10, 20, 30 µg/animal) incorporated in small unilammellar vesicles in presence or absence of cholesterol were inoculated into hamsters by intraperitoneal route and challenged with $1x10^7$ promastigotes 21 days after LPG challenge. Percent protection was calculated as mentioned above.

RESULTS:

LPG provides protection against *L. donovani* infection in susceptible golden hamsters:

The potential of LPG in protecting susceptible golden hamsters against *L. donovani* infection was studied by administration of a singe dose of 10 µg/animal of purified LPG alone or incorporation in various liposomised preparations, 21 days prior to infection. The percent protection against *L. donovani* infection in susceptible golden hamsters on pretreatment with LPG and its liposomised preparations is summarized in figure 1. Pretreatment of animals with LPG alone or in various liposomised form provided significant protection against *L. donovani* infection. The group of animals receiving LPG alone showed a relatively low protection of about 22 percent. This protective efficacy of LPG was found to be significantly enhanced on its incorporation into liposomes. For LPG incorporated in cholesterol containing multilamellar liposomes about 35 percent protection was observed. Incorporation of LPG in unilamellar vesicles enhanced the protecting efficacy to 41 percent. A maximum protection of about 48 percent was observed for LPG incorporated in cholesterol containing unilamellar liposomes, which is more than double of that observed for LPG alone. These observations suggest that small unilamellar vesicles (SUV) in presence of cholesterol responed better as compared to other preparations.

Optimization of conditions for pretreatment with LPG:

Effect of variation of pretreatment schedule with liposomized LPG on the protection against *L. donovani* infection in hamsters was studied. Figure 2 shows the protection compared to control observed in animals treated with a constant dose (10 µg/animals) on day 2, 10, 14, 21 and 28 prior to infection. A low protection of about 32 percent compared to control

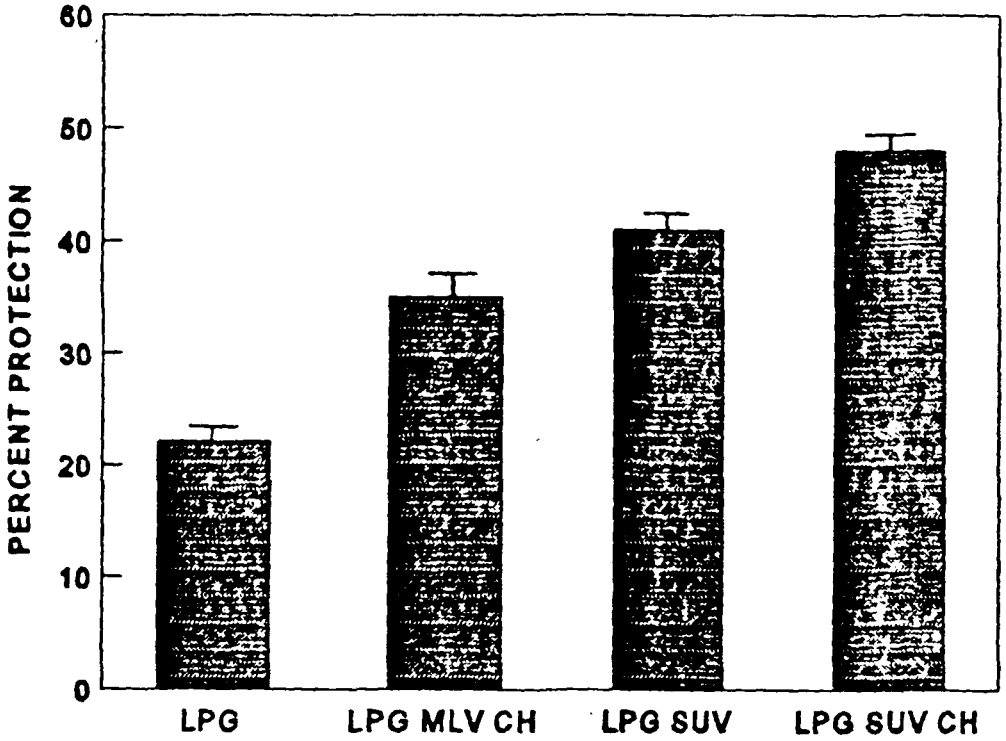

Figure 1: Protection against *L. donovani* infection in Syrian golden hamsters on pretreatment with LPG alone and its various liposomised forms. The LPG concentration in all the preparations was kept constant at 10 ug/animal. Values are represented as mean ±SE (n=20). The various abbreviations used are as follows: LPG MLV = LPG incorporated in cholesterol containing multilamellar vesicle; LPG SUV = LPG incorporated in unilamellar vesicle and LPG: SUVCH = LPG incorporated in cholesterol containing unilamellar vesicle.

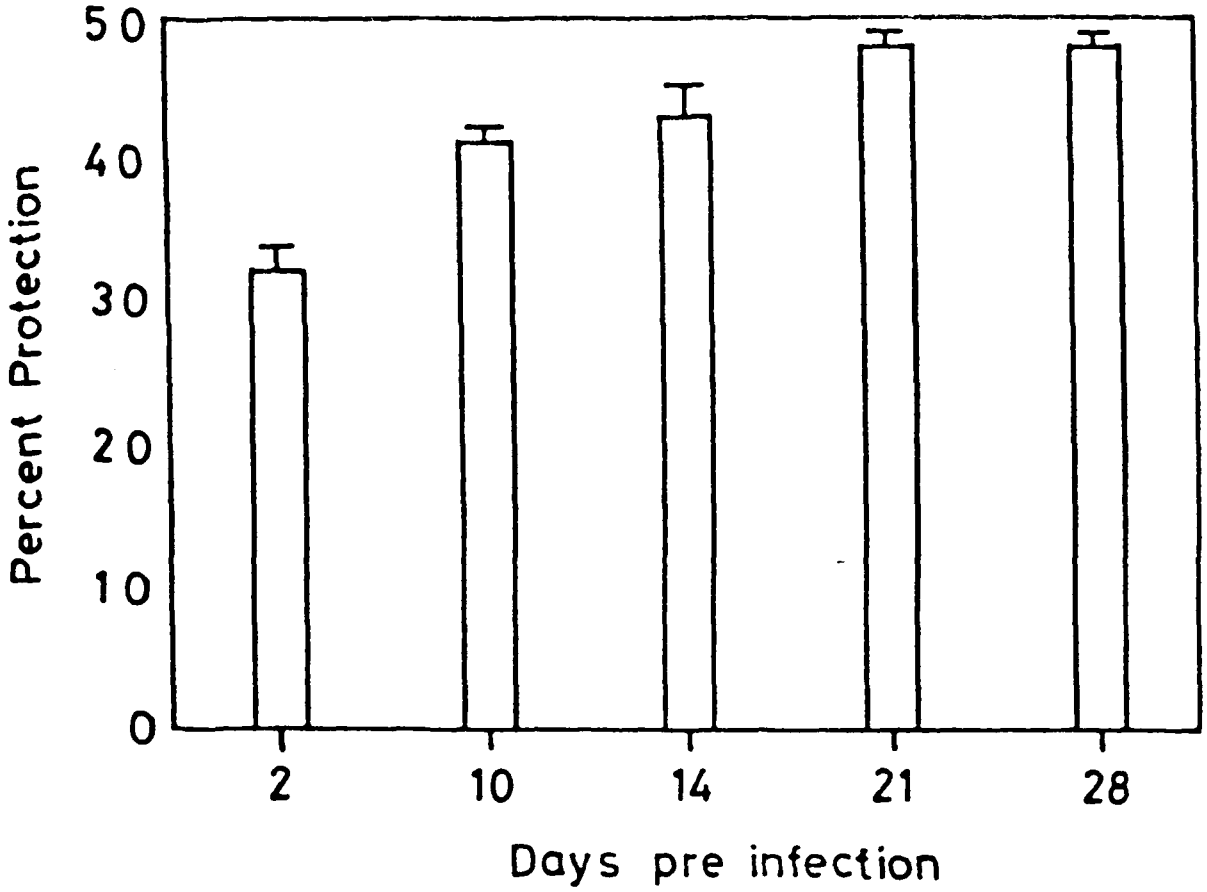

Figure 2: The effect of various pretreatment schedules using liposomised LPG (10 µg/animal) on the protection against *L. donovani* infection in susceptible golden hamsters. The protection was compared to control with treated animals on day 2-28 prior to infection. Values are represented as mean ± SE (n=15)

was observed for day 2 pretreatment with liposomised LPG. This was found to be enhanced significantly to about 41 and 48 percent on increasing the LPG pretreatment time to day 10 and 21, respectively, prior to infection. The results indicate that pretreatment of animals with liposomised LPG anywhere between 20 to 30 days is optimum for getting maximum protection against *L. donovani* infection.

The effect of multiple doses of LPG alone or its various liposomised preparations was also studied. Two doses 10 μg/ animal each of LPG was administered in a gap of 14 days. The results of the studies are summarized in Fig. 3. Although the booster dose of LPG incorporated in various liposomised preparations showed a slight enhancement in percent protection but almost no change was observed in case of LPG alone. The percent protection observed with liposomised preparations by the administration of booster dose was increased about 1.15 fold. The study suggests that single dose of LPG administration prior to challenge with promastigotes was sufficient in providing optimum protection against *L. donovani* infection.

Effect of intraperitoneal and intracardial administration of LPG preparations on efficacy was also studied. A higher percent protection by intracardial administration as compared to intraperitoneal route was observed. Although administration by intracardial route was found to provide better protection than intraperitoneal route but intraperitoneal route is preferred because the intracardial administration of any vaccine/chemoprophylactic agent is risky, difficult as well as it requires expertise hand to perform it. Furthermore, the intraperitoneal administration is easy to perform, well accepted by others and risk factors are low as compared to intracardial route.

Dose dependent LPG mediated protection against *L. donovani* infection in hamsters:

Studies on concentration dependence of liposomised LPG in protecting hamsters against *L. donovani* infection was carried out for optimization of dose required to give maximum protection. 10, 20 and 30 μg LPG was incorporated in liposomes and administered intraperitoneally in golden hamsters 21 days prior to infection. A dose dependent protection by liposomised UR6 LPG was observed (Table 1 and Figure 4). For increase in concentration of LPG incorporated in SUV from 10 to 20 μg/animal, an increase in percent protection from 50 to 68 percent was observed. Further increase of LPG concentration to 30 μg/animal showed no significant enhancement in percent protection. These result indicate that single administration of 20 μg of LPG incorporated in cholesterol containing SUV is the most effective dose for achieving maximum protection by UR6 LPG against *L. donovani* infection in susceptible golden hamsters.

Liposomes injected intravenously into the blood circulation are readily taken up by the mononuclear phagocyte system (Gregoriadis, 1988; Raz *et al.*, 1981; PerezSolar *et al.*,

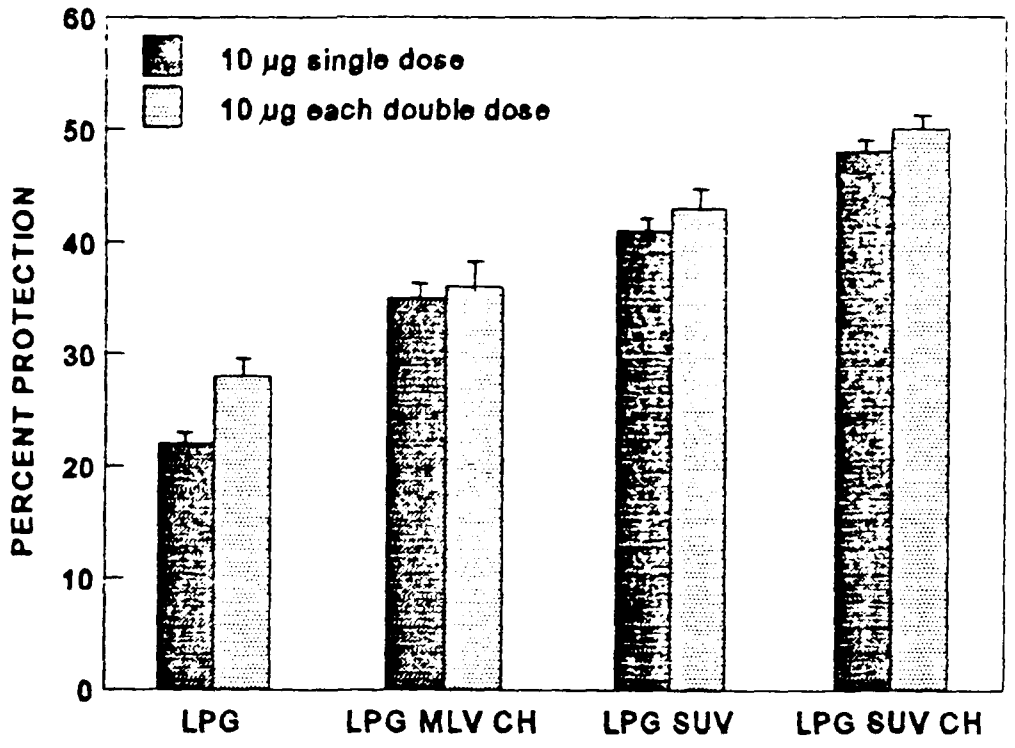

Figure 3: The effect of multiple doses of LPG alone or its various liposomised preparations. Susceptible golden hamsters were pretreated with LPG and its various liposomised preparations on day 14 prior to infection with *L. donovani* promastigotes of strain Dd8. The percent protection compared to control in these treated group of animals was calculated on day 45 post infection. Values are represented as mean ±SE (n=10). The various abbreviations used are as follows: LPG MLV = LPG incorporated in cholesterol containing multilamellar vesicle; LPG SUV = LPG incorporated in unilamellar vesicle and LPG SUVCH = LPG incorporated in cholesterol containing

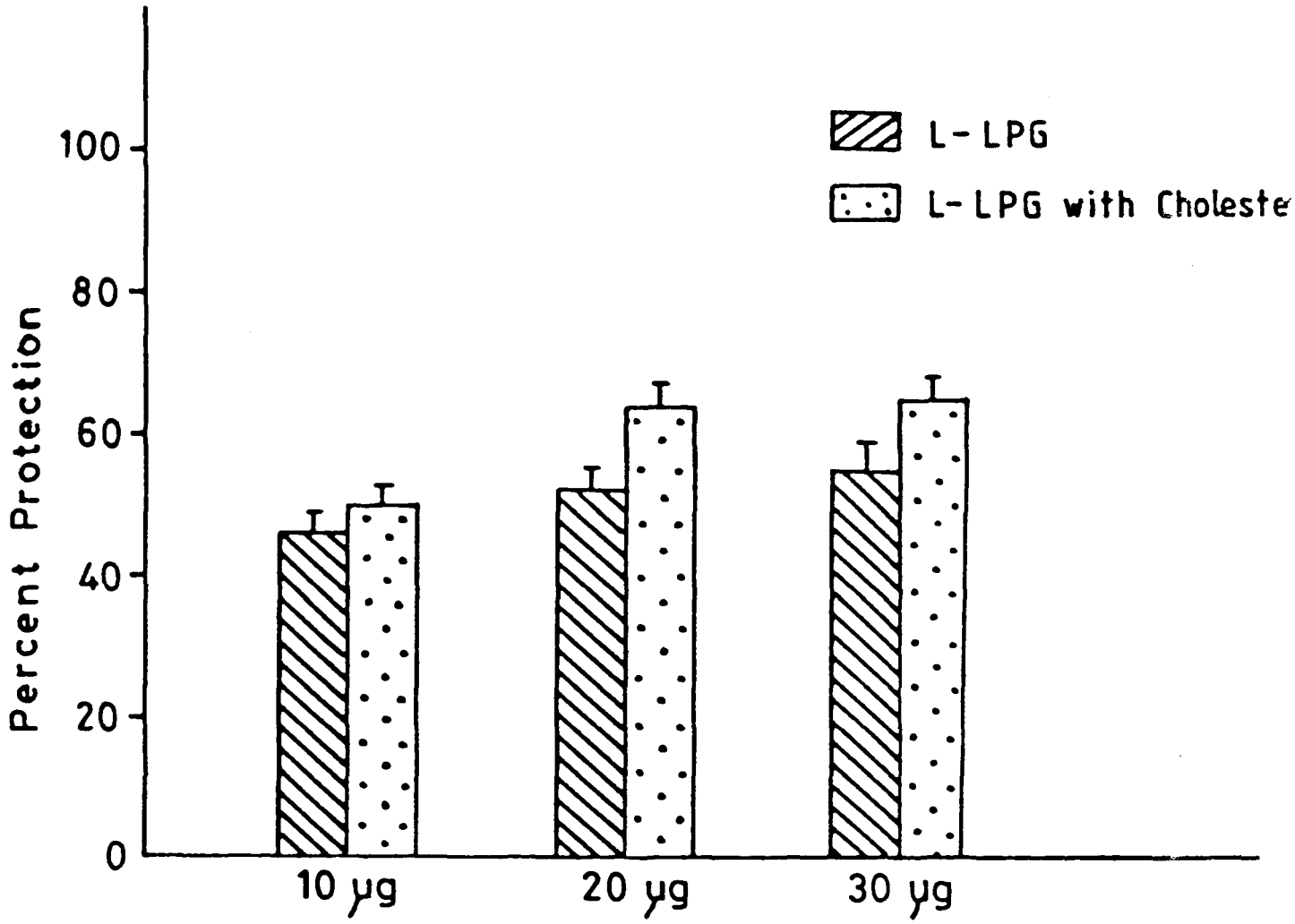

Figure 4: The protection against *L. donovani* infection in susceptible golden hamsters on pretreatment with various dose of liposomised LPG incorporated in SUV in presence and absence of cholesterol. Syrian golden hamsters were administered a single dose of desired concentration of LPG in SUV, 21 days prior to infection with *L. donovani* promastigotes of strain Dd8. The results presented were obtained on day 45 post infection by sacrificing animals. Values are represented mean ±SE (n=15). The various abbreviations used are as follows: LPG SUV = LPG incorporated in unilamellar vesicle and LPG SUVCH = LPG incorporated in cholesterol containing unilamellar vesicle.

TABLE 1:

Protection against *L. donovani* infection in susceptible syrian golden hamsters on pretreatment with varying dose of UR6 LPG incorporated in unilamellar liposome.

LPG concentration in liposomes	Mean number of amastigotes /100 splenic macrophages	Protection (%)
1. Control	25.38±3.2	-
2. 10 µg/animal	12.56±1.22	50
3. 20 µg/animal	8±0.92	68
4. 30 µg/animal	7.56±1.13	69

Values represent mean±SE (n=15).

Syrian golden hamsters were given single i.p. injection of desired concentration of LPG incorporated in cholesterol containing unilamellar liposomes 21 days prior to infection with *L. donovani* promastigotes of strain Dd8. The results were obtained on day 45 post infection by sacrificing animals.

1985). It has been suggested that negatively charged MLVs are more readily taken up by murine peritoneal macrophages (Mehta *et al.*, 1982) and human peripheral blood monocytes (Mehta *et al.*, 1984). Hence, studies using negatively and positively charged liposomes were also carried out. 20 and 30 μg of LPG were incorporated both in negatively charged (Dicetylphosphate) and positively charged (streaylamine) egg PC SUV and MLV liposomes. The results of these studies are summarized in Table 2. With negatively changed liposomes showedno significant change in percent protection as compared to neutral liposomes was observed.

DISCUSSION:

The importance of T-cell responses in recovery and resistance to leishmaniasis is well demonstrated. It is therefore, of considerable importance to use these responses to evaluate the immunostimulatory potential of *Leishmania* antigens. LPG is the major surface glycoconjugate of all *Leishmania* promastigotes and has been reported to be both immunogenic and antigenic. Hence the induction of immune responses using LPG might be an effective approach to inhibit parasite survival and replication in the mammalian host. LPG has been used successfully to immunize mice against cutaneous infection with *L. major* (Handman and Mitchell, 1985; McConville *et al*, 1987), as well as to elicit T-cell response in leishmaniasis patients (Kemp *et al.*, 1991; Mendonca *et al.*, 1991). Mice immunized with LPG produce LPG specific IgG and increases frequency of *L. major* reactive T-cells, although these cells do not respond to LPG *in vitro* (Moll *et al.*, 1989). Mendonca *et al.* (1991) have demonstrated that LPG stimulated PBMC in cutaneous leishmaniasis patients, but that treatment of LPG molecule with proteinase K abolished its stimulatory activity. It has been demonstrated that protein copurified with LPG was responsible for the observed T-cell stimulation (Russo *et al.*, 1992). LPG which is associated with protein contaminants elicited *in vitro* proliferative responses and IFN-γ production in cutaneous, mucocutaneous and cured visceral leishmaniasis patients. However, protein free LPG and repeating disaccharide units of LPG did not stimulate LPG specific T-cell (Russo *et al.*, 1992). Similarly, purified glycolipids are weak stimulator whereas, the protein associated glycolipid was found to be high stimulator of T-Cell response. A LPG associate protein (Mr 11000) was isolated and it was found that lymphocyte stimulation was associated with the protein component of LPG and not the glycan (Jardim *et al.*, 1991; 1995; Pimenta *et al.*, 1994).

After establishing that LPG from *L. donovani* promastigotes (strain UR6) inhibits attachment of promastigotes of strain Dd8 to macrophages and also provides protection to macrophages against *L. donovani* infection (Ali *et al.*, 1995), we set out to explore whether pretreatment of hamsters with UR6 LPG can provide protection against *L. donovani* infection. Pretreatment of hamsters with UR6 LPG or its liposomized preparation anywhere between 10 to

TABLE 2:

Protection against *L. donovani* infection in susceptible syrian golden hamsters on pretreatment with 20 μg/animal single administration of UR6 LPG alone or on incorporation in liposomes.

A: [a]Neutral liposomes

Groups	Mean number of amastigotes /100 splenic macrophages		Protection (%)	
	Day 45	Day 60	Day 45	Day 60
1. Control	26-2.2	41.22+2.8	-	-
2. LPG alone	19-1.7		27	
3. MLV	25-1.9		-	
4. MLV LPG	15.5-2.0	26.3+1.2	40	39
5. SUV	25+1.2		-	
6. SUV LPG	9.5-1.6	17.66+1.3	63	59

B: [b]Negatively charged liposomes:

Groups	Mean number of amastigotes 100 splenic macrophages		Protection (%)	
	Day 45	Day 60	Day 45	Day 60
1. Control	28-3.2	42.33+ 2.8	-	-
2. MLV	28-1.9		-	
4. MLV LPG	19.2+1.2	30.33+1.24	32	28
5. SUV	27.3+1.7		-	
6. SUV LPG	9.5+1.6	15.66+1.6	66	63

a: Egg phosphatidylcholine liposomes containing cholesterol.

b: Egg phosphatidylcholine liposomes containing cholesterol and dicetyl phosphate.

Values are represented as mean+SE (n=20).

The various abbreviations used are as follows: MLV= multilamellar liposomes; SUV= unilamellar liposomes; MLV LPG = LPG incorporated in cholesterol containing multilamellar liposomes and SUV LPG= LPG incorporated in unilamellar liposomes.

Susceptible syrian golden hamsters were pretreated with LPG and its various liposomised forms on day 21 prior to infection with L. donovani promastigotes of strain Dd8. The percent protection compared to control was calculated on day 45 (by spleen biopsy) and on day 60 post infection by killing the respective animals of the groups.

SUMMARY

Lipophosphoglycan (LPG) is a major cell surface glycoconjugate of *Leishmania* parasites. This unusual glycoconjugate is present throughout the various phases of growth in *Leishmaia* parasites. The promastigotes plasma membrane contains about 1.25 million copies/cell of LPG, accounting for atleast 25% of its cell wall. Structurally, LPG is composed of a neutral cap and a variable composition of a repeating phosphorylated disaccharide units attached via a conserved phosphosaccharide core to a phosphatidylinositol (PI) lipid anchor. All LPG molecules contain multiple unit of a backbone structure of PO_4-6-Gal(β 1–>4)Man α 1- .

LPG plays an important role in the biology of the parasite due to its surface location, its developmental regulation during the life cycle and the reduce virulence of the LPG-deficient organisms. A variety of functions and activities of LPG in the mammalian host have been experimentally demonstrated or suggested. These include, involvement in attachment and entry of promastigotes into mammalian macrophages, protection of parasite within phagolysosomal compartment and as a recognition molecule for the T-lymphocyte dependent immune responses characteristic of leishmaniasis. LPG is an inhibitor of protein kinase C, inhibitor of oxidative burst, inhibitor of viral fusion, signal transduction and scavenger of oxygen free radicals. Lipophosphoglycan has also been proposed to induce a protective immune response in mice and therefore, is considered to be a candidate vaccine against leishmaniasis.

In recent years, the promastigote cell surface has received considerable attention in view of its importance in interaction with the immune system and for parasite recognition, uptake and survival in macrophages. The cell surface glycoconjugate, lipophosphoglycan (LPG) has been isolated, purified and characterized from *Leishmania* parasites of different species. However, no attempt has been made to study the LPG from non-pathogenic strains. Hence, LPG from promastigotes of non-pathogenic strain UR6 was isolated purified, characterized and its potential as immunoprophylactic agent and/or candidate vaccine against experimental visceral leishmaniasis was evaluated. Isolation of LPG was carried out using different organic solvent mixtures. Crude LPG was purified by size-exclusion, and hydrophobic affinity chromatography. From 25.0 ml (27.0 gm) packed cells, about 150 mg of crude LPG was obtained. This on partial purification yielded 31 mg of partially purified LPG which on octyl Sepharose CL-4B column gave a yield of about 15.0 mg of pure LPG. Characterization of purified LPG was carried out by thin layer chromatography (TLC), SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and NMR spectroscopy. A single fused spot with R_f value similar to the LPG of pathogenic strains was observed on TLC. The purity of LPG molecule was assessed by SDS-PAGE followed by periodic acid schiff's (PAS) staining. On PAS staining of gel, LPG was the only carbohydrate staining species observed and it migrated as a single diffused band. The molecular weight of UR6 LPG. obtained by the

migration of standard proteins on SDS-PAGE, was found to be in the range of 20-25 kDa.

The protective potential of *L. donovani* LPG, against *L. donovani* infection in peritoneal macrophages has been studied. The studies demonstrate that UR6 LPG inhibits binding of promastigotes of Dd8 strain to peritoneal macrophages in a concentration dependent manner. A maximum inhibition of about 85-90% was observed for 1000 ng/ml of LPG. No further enhancement in inhibition with increase in LPG concentration upto 2000 ng/ml was observed. Similar results were also observed when LPG was incorporated into small unilamellar vesicle (SUV) containing cholesterol. Furthermore, UR6 LPG provided a significant protection (about 85-90%) in peritoneal macrophages against promastigotes infection.

In order to look into the possibility whether LPG mediated protection in macrophages system against *L. donovani* was either due to inhibition of attachment of promastigotes to macrophages or killing of amastigotes inside the macrophages, studies with pre and post treatment of macrophages with UR6 LPG were carried out. It was observed that the peritoneal macrophages which were treated with LPG before being challenged with promastigotes provided significant protection against *L. donovani* infection. However, those macrophages which were first challenged with promastigotes and then treated with LPG provided similar pattern of infection as observed that for control. These observations, suggest that pretreatment of peritoneal macrophages is necessary for achieving protection which is depend on the inhibition of attachment of promastigotes to peritoneal macrophages.

In vivo efficacy of LPG molecules against visceral leishmaniasis was evaluated in susceptible golden hamsters using LPG alone or incorporated in liposomes. Pretreatment of hamsters with LPG anywhere between 10-28 days prior to infection did provide significant protection against *L. donovani* infection. The protective efficacy of LPG was enhanced significantly on its incorporation in liposomes. LPG incorporated in cholesterol containing multilamellar liposomes provided 40 percent protection whereas LPG incorporated in small unilamellar vesicles (SUV) provided about 65-70 percent protection. This protection was more than double as compared to LPG alone. Furthermore, for incorporation of LPG in negatively charged liposomes a similar percent protection as that for neutral liposomes was observed. Studies on concentration dependence of liposomised LPG in protecting hamster against *L. donovani* infection showed that a single dose of 20 μg/animal of liposomised LPG incorporated in SUV provided a maximum protection of about 68 percent. The effectiveness of liposome incorporated LPG compared to LPG alone in protection against *L. donovani* in hamsters can be due to induction of an effective T-cell response. This possibility is indicated from the fact that increased percent protection was observed on increasing the pretreatment schedule with liposomised LPG from 2 days to 21 days.

BIBLIOGRAPHY

Acterberg, V., Gercken, G. (1987). Cytotoxicity of ester and ether lysophospholipids on *Leishmania donovani* promastigotes. *Mol. Biochem. Parasitol.* **23**, 117-122.

Adhya, S., Chatterjee, M., Hassan, M.Q., Mukherjee, S., Sen, S. (1995). Detection of *Leishmania* in the blood of early kala-azar patients with the aid of the polymerase chain reaction. *Trans. Roy. Soc. Trop. Med. Hyg.* **89**, 622-624.

Adler, S. and Theodor, O. (1927). The transmission of *Leishmania tropica* from antificially infected sandflies to man. *Ann. Trop. Med. Parasitol.* **21**, 89-111.

Aebischer, T. (1994). Recurrent cutaneous leishmaniasis: A role for persistent parasites. *Parasitol. Today*, **10** 25-27.

Afonso, L.C.C., Scharton, T.M., Vieira, L.Q., Wysocka, M., *et al* (1993). The adjuvant effect of interleukin-12 in a vaccine against *L. major. Sci.* **263**, 235-237.

Akiyama, H.J. and Tayor, J. C. (1970). Effect of macrophage engulfment and temperature on the transformation process of *L. donovani Am. J. Trop. Med. Hyg.* **19**, 249-254.

Alexander, J., Vickerman, K. (1975). Fusion of host cell secondary lysosomes with the parasitophorous vacuoles of *Leishmania mexicana* infected macrophages. *J. Protozool.* **22**, 502-508.

Alexander, J. and Russell, D.G. (1992). The interaction of *Leishmania* species with macrophages. *Adv. Parasitol.* **32**, 175-254.

Ali, V., Mehrotra, S., Singha, U.K. and Bhakuni, V. (1995). Isolation, purification of lipophosphoglycan from *Leishmania donovani* promastigotes of a nonpathogenic strain and its *in vitro* efficacy in protecting against *leishmania donovani* infection. *J. Parasitic. Dis.* **19**, 153-158.

Anderson, S., David, J.R. and McMahon-Pratt. D. (1983). *In vivo* protection against *Leishmania mexicana* mediated by monoclonal antibodies. *J. Immunol.* **131**, 1616-18.

Angus, W. and Lester, R.L. (1972). Turnover of inositol and phosphorus containing Lipids in *Saccharomyces cerevisiae*: Extracellular accumulation of glycerophosphorylinositol derived from phosphatidylinositol. *Arch. Biochem. Biophys.* **151**, 483-495.

Anon. (1990). WHO *Tech. Rep. Ser.* No. 793.

Antoine, J.C. (1995). Co-stimulatory activity of *Leishmania* infected macrophages. *Parasitol. Today* **11**, 242-243.

Antunes, C.M.F., Mayrink, W., Magalhaes, P.A., Costa, A., *et al* (1986). Controlled field trials of a vaccine against New World cutaneous leishmaniasis. *Int. J. Epd.* **15**, 572-580.

Arnot, D.E., and Barker D.C. (1981). Biochemical identification of cutaneous leishmaniasis by analysis of kinetoplast DNA. Sequence homologies in *Leishmania* kDNA. *Mol. Biochem. Parasitol.* **3**, 47-56.

Ashford, R.W., Desjaex, P., and DeReath, P. (1992). Estimation of population at risk of infection and number of cases of *Leishmaniasis. Parasitol. Today* **8**, 104.

Ashley, J.N., Barber, J., Ewins, A.J., Newberry, G.S., Self, A.D.H. (1942). A chemotherapeutic comparision of the trypanocidal action of some aromatic diamidines. *J. Chem. Soc.* Part I, 103.

Ashley, J.N. and Harris, H.W. (1946). Attempts to find new chemotherapeutic amidines Part III. Nuclear substituted derivatives of 4,4 and 3,3-diamidine stiloamine. *J. Chem. Soc.* **11**, 567.

Aukerman, S.L., Young, J.D. Chang, K.T. and Ralph, P. (1990). Preclinical pharmacology of recombinant human MG-CSF. *J. Leukoc Biol.* **97**.

Avila, J.L., Rojas, M., Garcia, L. (1988). Persistence of elevated levels of galactosylα(1-->3) galactose antibodies in sera from patients cured of visceral leishmaniasis. *J. Clin. Microbiol.* **26**, 1842-1847.

Avila, J.L., Rojas, M., Towbin, H. (1988). Serological activity against galactosyl-α(1-->3) galactose in sera from patients with several kinetoplastida infections. *J. Clin. Microbiol,* **26**, 126-132.

Badaro, R., Johnson, W. (1993). The role of interferon-γ in the treatment of visceral and diffuse cutaneous leishmaniasis. *J. Infect. Dis.* **167**, (suppl.) 13-17.

Badaro, R., Nascimenoto, C., Carvalho, J.S., Badaro, F., Ho, J.L., Reed, S.G. and Johnson, Jr. W.D. (1994). Recombinant human granulocyte-macrophage colony-stimulating factor reverses neutropenia and reduces secondary infections in visceral leishmaniasis. *J. Infect. Dis.* **170**, 413.

Badaro, R., Nascimento, C., Carvalho, J.S., Badaro, F., Russo, D., *et al.* (1994). Recombinant human granulocyte-macrophage colony-stumulating factor reverses neutropenia and reduces secondary infections in visceral leishmaniasis. *J. Infect. Dis.* **170**, 413-415.

Badaro, R., Nascimento, J.S., *et al* (1995). Recombinant human granulocyte-macrophage colony-stimulating factor reverses neutropenia and reduces secondary infections in visceral leishmaniasis. *Ann. Trop. Med. Parasitol.* **89** 114.

Bahar, K., Shidani, B., Dowlati, Y., *et al* (1993). Overview of human vaccine studies using killed *L. major* in 13th International Congress for Tropical Medicine and Malaria Faculty of Tropical Medicine. Mahidol University, Bangkok, **1**, 183.

Bahr, V., Stierhof, Y.D., Ilg, T., Demar, M., Quinten, M. and Overath, P. (1993). Expression of lipophosphoglycan, high molecular weight phosphoglycan and glycoprotein 63 in promastigotes and amastigotes of *Leishmania mexicana*. *Mol. Biochem. Parasitol.* **58**, 107-122.

Barker, D.C., De Brujn, M., Eresh, S. and Smyth, A. (1991). Diagnosis of leishmaniasis using PCR on parasite DNA extracted from human biopsy samples, aspirates, sandflies and cultures. *Memorias da Instituto Osrraaldo Cruz* **86**, 73-74.

Barker, D.C. (1989). Molecular approaches to DNA diagnosis. *Parasitol.* **99**, S125-S146.

Bates, P. (1993). Axenic culture of *Leishmania donovani* in axenic culture. *Parasitol.* **108**, 1-9.

Bates, P.A., Hermes, I., Dwyer, D.M. (1990). Golgi-mediated post-translational processing of secretory acid phosphatase by *Leishmania donovani* promastigoes. *Mol. Biochem. Parasitol.* **38**, 247-256.

Bates, P.A. (1994). Complete developmental cycle of *Leishmania mexicana* like forms. *Parasitol.* **105**, 193-202.

Bates, P.A. (1995). The lipophosphoglycan-associated molecules of *Leishmania. Parasitol. Today*, **11**, 317-318.

Belosevic, M., Finbloom, D.S., Van der Meide, P.H., Slayter, M.V. and Nacy, C. A. (1989). Administration of monoclonal anti-IFN-γantibodies *in vivo* abrogates natural resistance of C3H/HeN mice to infection with *Leishmania major. J. Immunol.* **143**, 266-274.

Berens, R.L., Brun, R. and Krassner, S.M. (1976). A simple monophasic medium for axenic culture of haemoflagellates. *J. Parasitol.* **62**, 360-365.

Berman, J.D. and Dwyer, D.M. (1981). Expression of *Leishmania* antigen on the surface membrane of infected human macrophages *in vitro. Clin. Exp. Immunol.* **44**, 342-348.

Berman, J.D. (1988). Chemotherapy for leishmaniasis biochemical mechanism, clinicl efficacy and future strategies. *Rev. Infect. Dis.* **10**, 560.

Berman, J.D. (1992). Activity of amphotericin B cholesterol dispersion (Amphocil) in experimental visceral leishmaniasis. *Antimicrob. Agents. Chemother.* **36**, 1978-1980.

Berridge, M.J. (1984). Inositol triphosphate and diacylglycerol as second messengers. *Biochem. J.* **220**, 345-360.

Beverley, S.M. and Truco, S.J. (1995). Identification of genes mediating lipophosphoglycan biosynthesis by functional complementation of *Leishmania donovani* mutants. *Ann. Trop. Med. Parasitol.* **89**, 11-17.

Bhakuni, V., Singha, U.K., Dutta, G.P., Levy, H.B. and Maheshwari, R.K. (1996). Killing of *Leishmania donovani* amastigotes by Poly IC:LC in hamsters. *J. Interferon. Cyt. Res.* **16**, 321-325.

Bhatnagar, S., Guru, P.Y., Katiyar, J.C., Srivastava, R., Mukherjee, A.P., Akhtar, M.S., Seth, M.,

Bhaduri, A.M. (1989). Exploration of antileishmanial activity in heterocycles; results of their *in vivo* and *in vitro* bioevaluations. *Ind. J. Med. Res.* 89, 439.

Bhattacharya, A., Prasad, R. and Sacks, D.L. (1992). Isolation and partial characterization of a lipophophosglycan from a pathogenic strain of *Entamoeba histolytica*. *Mol. Biochem. Parasitol.* 56, 161-168.

Bianco, C., Griffin, F.M.Jr. and Silverstein, S.C. (1975). Studies of the macrophage complement receptor. Alternatives of receptor function and macrophage activation. *J. Exp. Med.* 141, 1278-1290.

Biegel, D., Topper, G., Rabinovitz, M. (1983). *Leishmania mexicana* terperature sensitivity of isolated amastigotes and of amastigotes infecting macrophages in culture. *Exp. Parasitol*, 56, 289-97.

Black C.D.V., Watson, G.J. and Ward, R.J. (1977). The use of Pentostam liposomes in the chemotherapy of experimental leishmaniasis. *Trans. Roy. Soc. Trop. Med. Hyg.* 71, 550-552.

Blackwell, J.M., Ezekowitz, R.A.B., Roberts, M.B., Channon, J.Y., Sim., R.B., Gordon, S. (1985). Macrophage complement and lectin-like receptors bind Leishmania in the absence of serum. *J. Exp. Med.* 162, 324-331.

Bogdan, C., Gessner, A. and Rollinhoff, M. (1993). Cytokines in *Leishmaniasis*: a complex network of stimulatory and inhibitory interactions. *Immunobiol.* 189, 356-396.

Bogdan, C., Streck, H., Rollinghoff, M. and Solbach. W. (1989). Cyclosporin A enhances elimination of intracellular. *L. major* parasites by murine macrophages. *Clin. Exp. Immunol.*75, 141-146.

Bogdan C., Vodovotz, Y. and Nathan, C. (1991). Macrophage deactivation by interleukin 10. *J. Exp. Med.* 174, 1549.

Bordier, C. (1987). The promastigote surface protease of *Leishmania*. *Parasitol. Today* 3, 151-153.

Borovsky, D. and Schlein, Y. (1987). Trypsin and chymotrypsin-like enzymes of the sandfly *Phlebotomus papatasi* infected with *Leishmania* and their possible role in vector competence. *Med. Vet. Entomol.* 1, 235-242.

Bouvier, J., Bordier, C., Vogel, H., Reichelt, R. and Etges, R. (1989). Characterization of the promastigote surface protease of *Leishmania* as a membrane-bound zinc endopeptidase. *Mol. Biochem. Parasitol.* 37, 235-246.

Bouvier, J., Etges, R.J., Bordier, C. (1985). Identification and purification of membrane and soluble forms of the major surface promastigotes *J. Biol. Chem.* 260, 15504-509.

Bouvier, J., Etges R.J., Bordier, C. (1987). Identification of the promastigote surface protease in seven species of *Leishmania*. *Mol. Biochem. Parasitol.* 24, 73-79.

Bouvier, J., Schneider, P., Etges, R. and Bordier, C. (1990). Peptide substrate specificity of the membrane bound metalloprotease of *Leishmania*. *Biochem.* **29**, 10113-10119.

Boysia, F.T. (1968). Delayed hypersensitivity to leishmanial antigens in the guinea pig. Ph.D. Thesis, Rutgers. The state university, New Brunswick, N.J. 139.

Bravo, R., Neuburg, M., Burckhardt. J., Almendral. J., Wallich, R., *et al.*, (1987). Involvement of common and cell type specific pathways in C-fos gene control: Stable induction by cAMP in macrophages. *Cell* **48**, 251-260.

Brunda, M.J., Luistro, L., Warrier R.R. *et al* (1993). Antitumor and antimetastatic activity of interleukin 12 against murine tumors. *J. Exp. Med.* **178**, 122-130.

Bryceson, A.D.M., Bray, R.S., Wolstencroft, R.A. and Dumonde, D.C. (1970). Immunity in cutaneous leishmaniasis of guinea pig. *Clin. Exp. Immunol.* **7**, 301-341.

Bryceson, A.D.M. (1970). Diffuse cutaneous leishmaniasis in Ethiopia III Immunological studies. *Trans.Roy. Soc. Trop. Med. Hyg.* **64**, 380-387.

Bryceson, A.D.M. (1987). Therapy in man *in*: "The Leishmaniasis in Biology and Medicine" (W. Peters and R. Killick-Kendrick, eds) Vol. 2, 848-907. Academic Press, London.

Buchmuller-Rouiller, Y. and Mauel, J. (1987). Impairment of the oxidative metabolism of mouse peritoneal macrophages by intracellular *Leishmania* spp. *Infec. Immun.* **55**, 587-593.

Butler, P.G. (1978). Levamisole therapy of chronic *Leishmania tropica*. *J. Trop. Med. Hyg.* **81**, 221.

Button, L.L., Reiner, N.E. and McMaster, W.R. (1991). Modification of GP63 genes from diverse species of *Leishmania* for expression of recombinant protein at high levels in *Escherichia coli*. *Mol. Biochem. Parasitol.* **44**, 213-24.

Button, L.L., Wilson, G., Astell, C.R. and McMaster, W.R. (1993). Recombinant *Leishmania* surface glycoprotein GP63 is secreted in the baculovirus expression system as a latent metalloproteinase. Gene **134**, 75-81.

Button, L.L. and McMaster, W.R. (1990). Molecular cloning of the major surface antigen of *Leishmania*. *J. Exp. Med.* **171**, 599-604.

Camara, M., Ortiz, G. Valero, P.L., Molina, R., Navarro, I., Chance, M.L. and Segovia, M. (1995). Complement-mediated lysis and infectivity for mouse macrophages and sandflies of virulent and attenuated *Leishmania major* promastigotes varying in expression of the major surface protease and lipophosphoglycan. *Ann Trop. Med. Parasitol.* **89**, 243-251.

Cantella, R.A. (1968). Leishmaniasis and nucleic acid: Immunological approach. M.S. Thesis,

Rutgers. University, New Brunswick, N.J. 103.

Cappai, R., Morris, L., Aebischer, A., Bacic, A., Courtis, J., Kellether M., McLeod, K., Moody, S.F., Osborn, A.H. and Handman, E. (1994). Ricin-resistant mutants of *Leishmania major* cause lesions in BALB/c mice. *Parasitology*. **108**, 397-405.

Carvalho, E.M., Bardaro, R., Reed, S.G. Jones, T.C. and Johnson, W.P. (1985a). Absence of gamma interferon and interleukin 2 production during active visceral leishmaniasis. *J. Clin. Invest.* **76**, 2066-2069.

Carvalho, E.M., Johnson, W.D., Barreto, E., Marsden, P.D., Costa, J.L.M., Reed, S. and Rocha, H. (1985b). Cell mediated immunity in American cutaneous and mucosal leishmaniasis. *J. Immunol.* **135**, 4144-4148.

Carver, M.A. and Truco, S.J. (1992). Biosynthesis of lipophosphoglycan from *L. donovani* Characterization of mannosyl transferase *in vitro*. *Arch. Biochem. Biophys.* **295**, 309-317.

Carver, M.A. and Turco, S.J. (1991). Cell free biosynthesis of lipophosphoglycan from *L. donovani* characterization of microsomal galactosyl transferase and mannosyl transferase activities. *J. Biol Chem.* **266**, 10974-10981.

Castes, M., Blackwell, J., Trujillo, D., Formica, S., Cabrera, M., Zorrilla, G., *et al* (1994). Immune response in healthy volunteers vaccinated with killed leishmanial promastigotes plus BCG I. Skin test reactivity T cell proliferation and interferon- production. *vaccine* **12**, 1041-1051.

Castes, M., Moros, Z., Martinez, A., Trijillo, D., Castellanos, P.L., Rondon, A.J. and Convit, J. (1989). Cell-mediated immunity in localised cutaneous leishmaniasis patients before and after treatment with immunotherapy or chemotherapy. *Parasite Immunol*, **11**, 211-222.

Chakraborty, P. and Das, P.K. (1988). Role of mannose N-acetyl glucosamine recepotrs in blood clearance and cellular attachment of *Leishmania donovani*. *Mol. Biochem. Parasitol.* **28**, 55-62.

Champsi, J. and McMahon-Pratt, D. (1988). Membrane glycoprotein M-2 protects against *Leishmania amazonensis* infection. *Infect. Immun.* **56**, 3272-3279.

Chan, S.H., Perussia, B., Gupta, J.W., Kobayashik M., Pospisil, M., Young, H.A., Wolf, S.F., Young, D., Clark, S.C. and Trinchieri, G. (1991). Induction of interferon-γproduction by natural killer cell stimulatory factor: characterization of the responders cells and synergy with other inducers *J. Exp. Med.* **173**, 869-880.

Chan, J., Fujira, T., Brennan, P., McNeel, M., Turco, S.J., *et al* (1989). Microbial glycolipids: Possible virulence factors that scavenge oxygen radicals. *Proc. Natl. Acad. Sci. USA*. **86**, 2453-2457.

Chance, M.L., Peters, W. and Shchovy, L. (1974). Biomedical toxonomy of *Leishmania I.* Observations on DNA. *Ann. Trop. Med. Parasitol.* **68**, 307-316.

Chance, M.L. (1995). New developments in the chemotherapy of *Leishmaniasis*. *Ann. Trop. Med. Parasitol.* **89**, 37-43.

Chang, C.S. and Chang, K.P. (1986). Monoclonal antibody affinity purification of a *Leishmania* membrane glycoprotein and its inhibition of *Leishmania* macrophage binding. *Proc. Natl. Acad. Sci. USA* **83**, 100-104.

Chang, K.P., Dwyer, D.M. (1976). Multiplication of a human parasite *Leishmania donovani* in phagolysosomes of hamster macrophages *in vitro*. *Science* **193**, 678-680.

Chang, K.P., Frong, O. and Bray, R.S. (1985). Biology of *Leishmania* and leishmaniasis, Vol. 1. Elsevior Biomedical Press, Amsterdam.

Chang, K.P. and Hendricks, A.L. (1985). Laboratory cultivation and maintenance of leishmania. In leishmaniasis, Edited by K.P. Chang and R.S. Bray, Elsevier, Amsterdam, New York, Oxford, 214.

Chang, K.P. (1979). *Leishmania donovani*: Promastigote-macrophage surface interaction *in vitro*. *Exp. Parasitol.* **48**, 175-189.

Chang, K.P. (1983). Cellular and Molecular mechanisms of intracellular symbiosis in Leishmaniasis. *Int. Rev. cytol. Suppl.* **14**, Academic Press Inc.

Change, K.P., and Dwyer, D.M. (1978). *L. donovani*: In hamster macrophage interaction *in vitro*. Cell entry, intracellular survival and multiplication. *J. Exp. Med.* **147**, 515-529.

Chatterjee, K.D. (1957). In: Parasitology, Edited by K.D. Chatterjee, Eleventh Edition, pp 54.

Chaudhuri, G., Chaudhuri, M., Pan, A. and Chang, K.P. (1989). Surface acid proteinase (gp63) of *Leishmania mexicana*: a metallo-enzyme capable of protecting liposome-encapsulated proteins from phagolysosomal degradation by macrophages. *J. Biol. Chem.* **264**, 7483-7489.

Cheesebrough, M. (1989). Leishmanial parasites in medical laboratory. Manual for tropical countries. **1**, 272.

Cherwinki, H.M. Schumacher, J.H. Brown, K.D. and Mosmann, T.R. (1987). Two types of mouse helper T-cell clone. III: Further differences in lymphokine synthesis between Th_1 and Th_2 clones revealed by RNA hybridization functionally monospecific bioassays and monoclonal antibodies. *J. Exp. Med.* **166**, 1229-1244.

Chulay, J.D., Flechenstem, L. and Smith, D.H. (1988). Pharmacokinetics of antimony during treatment of visceral leishmaniasis with sodium stibogluconate and meglumine antimoniate. *Trans. Roy. Soc. trop. Med. Hyg.* **82**, 69.

Chulay, J.D., Spencer, H.C. and Mugambi, M. (1985). Electrocardiographic changes during treatment of leishmaniasis with pentavalent antimony (Sodium stibogluconate). *Am J. Trop. Med.*

Hyg. **34**, 702.

Chuley, J.D., Bhatt, S.M. *et al* (1983). A comparison of three dosage regimens of sodium stibogluconate in the treatment of visceral leishmaniasis in Kenya. *J. Infect. Dis.* **148**, 148-155.

Chung, C.N., Gachihi, G., Muigai, R., Wasuinn, K. and Others (1985). Visceral leishmaniasis unresponsive to antimonial drugs.III successful treatment using a combination of sodium stibogluconate plus allopurinol. *Trans. Roy. Soc. Trop. Med. Hyg.* **79**, 715.

Cillari, E., Liew, F.Y. and Lelchuk, R. (1986). Suppression of IL-2 production by macrophages in genetically susceptible mice infected with *Leishmania major*. *Infect. Immun.* **54**, 386-394.

Clayton, C.E. and Mowatt, M.R. (1989). The procyclic acidic repetitive proteins of *trypanosoma brucei*: Purification and post-translational modification. *J. Biol. Chem.* **264**, 15088-15093.

Clinton, B.A., Stuber, L.A. and Palezuk, N.C. (1969). *L. donovani*: Atibody response to chicken ovalbumin by infected golden hamster. Exp. Parasitol. **25**, 171-180.

Clinton, B.A. (1969). *L. donovani* antibody production by infected hamsters and immunochemical studies of flagellate antigens. Ph.D. Thesis, Rutgers, The State University, New Brunswick, N.J. 155.

Collins, M., Baillie, A.J. and Carter, K.C. (1992). Visceral leishmaniasis in the BALB/c mouse: sodium stibogluconate treatment during acute and chronic stages of infection II. Changes in tissue drug distribution. *Int. J. Pharma.* **83**, 251-256.

Connell, N.D., Medina-Acosta, H., McMaster, W.R., Bloom, B.R., and Russell, D.G. (1993). Effective immunization against cutaneous leishmaniasis with recombinant bacille Calmette-Guerin expressing the *Leishmania* surface proteinase gp63. *Proc. Natl. Acad. Sci*, USA **90**, 1473-11477.

Convit, J., Rondon, A., Ulrich, M., Bloom, B., Castellanos, P.L. *et al* (1987). Immunotherapy versus chemotherapy in localized cutaneous leishmaniasis. *Lancet. i.* 401-404.

Cook, J.A., Holbrook, T.W. and Parker, B.W. (1980). Visceral leishmaniasis in mice: protective effect of glucan. *J. Reticuloendol. Sci.* **27** 567-573.

Criz, A., Coburn, C.M. and Beyerley, S.M. (1991). Double targeted gene replacement for creating null mutants. *Proc. Natl. Acad. Sci.* USA, **88**, 7170-7174.

Cross, G.A.M. (1990a) Glycolipid anchoring of plasma membrane proteins. *Annu. Rev. Cell. Biol.* **6**, 1-39.

Cross, G.A.M. (1990b). Cellular and genetic aspects of antigenic variation in typanosomes. *Ann. Rev. Immunol.* **8**, 83-100.

Cruz, A., Coburn, C.M., and Beverley, S.M. (1991). Double targeted gene replacement for creating null mutants. *Proc. Natl. Acad. Sci.* USA, **88**, 7170-7174.

Curry, A.J. and Kaye, P.M. (1992). Recomomant interleukin-1α-augments granuloma formation and cytokine production but not parasite clearance in mice infected with *Leishmania donovani*. *Infect. Immun.* **60**, 44222-4426.

Da Silva, R. and Sacks, D.L. (1987). Metacyclogenesis is a major determinant of *Leishmania* promastigote virulence and attenuation. *Infect. Immun.* **55**, 2802-2806.

Da Silva, R.P., Hall, B.F., Joiner, K.A. and Sacks, D.L. (1989). CR1, the C3b receptor, mediates binding of infective *Leishmania major* metacyclic promastigotes to human macrophages. *J. Immun.* **143**, 617-623.

Davidson, R.N., DI, Martno, L., Gradoni, L., Glaccno, R., Russo, R., Gaeta, G.B. *et al* (1994). Liposomal amphotericin B (AmBisome) in Mediteranean visceral leishmaniasis: a multi-centre trial *Qr. J. Med.* **87**, 75-81.

Davidson, R.N., diMartino, L., Gradoni, L. *et al.* (1996) Short-course treatment of visceral leishmaniasis with liposomal amphotericin-B (AmBisome). *Clin. Infect. Dis.* **22**, 938-943.

Davies, C.R., Cooper, A.M., Peacock, C., Lane, R.P., Blackwell, J.M. (1990). Expression of LPG and glycoprotein 63 by different developmental stages of *Leishmania major* in the sandfly *Phlebotomus papatasi*. *Parasitol.* **101**, 337-343.

Davis, C.E., Belosevic, M., Meltzer, M.S. and Nacy, C.A. (1988). Regulation of activated macrophage antimicrobial activities. Cooperation of lymphokines for induction of resistance to infection. *J. Immun.*. **141**, 627-635.

De Brujn, M.H.L., Labrada, L.A., *et al* (1993). A comparative study of diagnosis by the polymerase chain reaction and by current clinical methods using biopsies from Colombian patients with suspected leishmaniasis. *Trop. Med. Parasitol.* **44**, 201-207.

De Brujn, M.H.L. and Barker, D.C. (1992). Diagnosis of New World leishmaniasis: specific detection of species of the *Leishmania braziliensis* complex by amplification of kinetoplast DNA. *Adv. Trop.* **52**, 45-58.

De Ibarra, A.A.L., Howard, J.G., Snary, D. (1982). Monoclonal antibodies to *Leishmaia tropical major*: specificities and antigen location. *Parasitiol.* **85**, 523-531.

De Waal Malefyt, R., Haanen, J., Spits, J. *et al.* (1991). Interleukin 10 (IL-10) and viral IL-10 strongly reduce antigen-specific human T cell proliferation by diminishing the antigen-presenting capacity of monocytes via down regulation of class II major histocompatability complex expression. *J. Exp. Med.* **174**, 915.

Descoteaux, A., Luo, Y., Turco, S.J., Beverley, S.M. (1995). A specialized pathway affecting virulence glycoconjugates of *Leishmania*. *Sci.* 269, 1869-1872.

Descoteaux, A., Matlashewski, G. and Turco, S.J. (1992). Inhibition of macrophage protein kinase c-mediated protein phosphorylation by *L. donovani* lipophosphoglycan. *J. Immunol.* 149, 3008-3015.

Descoteaux, A., Turco, S.J., Sacks, D.L., Matlashewski, G., (1991). *Leishmania donovani* Lipophosphoglycan selectivity inhibits signal trasduction in macrophages. *J. Immunol.* 146, 2747-2753.

Descoteaux, A. and Turco, S.J. (1993). The lipophosphoglycan of *Leishmania* and macrophage protein kinase C. *Parasitol Today*, 9, 468-471.

Dewald, B., Thelen, M., Baggiolini, M., (1988). Two transduction sequences are necessary for neutrophil activation by receptor agonists. *J. Biol. Chem.* 263, 16179-16184.

Dietze, R., Fagundes, S.M.S., Brito, E.F., Milan, E.P. Fietosa, T.F. *et al* (1995). Treatment of kala-azar in Brazil with Amphocil (amphecericin B cholesterol dispersion) for 5 days. *Trans. Roy. Soc. Trop. Med. Hyg.* 89, 309-311.

Dietze, R., Milan, E.P., Berman, J.D., Grogl, M., *et al* (1993). Treatment of Brazilian kala-azar with a short course of Amphocil (amphotericin B cholesterol dispersion). *Clin. Infect. Dis.* 17, 981-986.

Dillon, R.J. and Lane, R.P. (1993). Influence of *Leishmania* infection on blood-meal digestion in the sandflies *Phlebotomus papatasi* and *P. langeroni*. *Parasitol. Res.* 79, 492-496.

Doran, T.I., and Herman, R. (1981). Characterization of populations of promastigotes of *Leishmania donovani*. *J. Protozool.* 28, 345-350.

Doyle, P.S., Engel, J.C., Pimenta, P.F., da Silva, P.P., Dwyer, D.M. (1991). *Leishmania donovani* long term culture of axenic amastigotes at 37°C. *Exp. Parasitol.* 73, 326-34.

Dubois, M., Gilles, K.A., Hamilton, J.K., and Fred Smith (1956). Colorimetric method for determination of sugars and related ubstances. Anal. Chem. 28, 350-355.

Dwyer, D. (1974). Lectin binding saccharides on a parasitic protozoa. *Sci.* 184, 471-472.

Dwyer, D. (1980). Isolation and partial characterization of surface membranes from *leishmania donovani*. *J. Protozool.* 27, 176-182.

Dwyer, D.M. (1977). *Leishmania donovani*: Surface membrane carbohydrates of promastigotes. *Exp. Parasitol.* 41, 341-358.

Ebert, F., Enriquez, G.L. Muhlpfordt, H. (1976). Electron Microscopic studies of the phagocytosis *L. donovani* by hamster peritoneal macrophages and its lysosomal activity *in vitro*. Behring Inst. Mitt. **60**, 65-74.

Eilam, Y., El-on, J., Spira, D.T. (1985). *Leishmania major*: excreted factor, calcium ions, and the survival of amastigotes. *Exp. Parasitol.* **59**, 161-168.

Elhay, M., Kelleher, M., Bacic, A., McConville, M.J., Tolson, D.L. *et al.* (1990). Lipophosphoglycan expression and virulence in ricin-resistant variants of *L. major*. *Mol. Biochem. Parasitol.* **40**, 255-268.

Elhay, M.J., McConville, M.J., Handman, E. (1988). Immunochemical characterization of a glycoinositol phospholipid membrane antigen of *L. major*. *J. Immunol.* **141**, 1326-1331.

El-Amin, E.R., Wright, E.P., Rahman, A.M., Kolk, A.J., Laarman, J.J. and Pondman, K.W. (1986). Serodiagnosis of Sudanese visceral and mucosal leishmaniasis: comparison of ELISA, immunofluorescence and indirect haemagglutination, *Trans. Roy Soc. Trop. Med. Hyg.* **80**, 271-274.

El-on, J., D.J. Bradley and J.C. Freeman. (1980). *Leishmania donovani*: Action of excreted factor on hydrolytic enzyme activity of macrophages from mice with genetically different resistance to infection. *Exp. Parasitol.* **49**, 167-174.

El-on, J., Schnur, L.F., Greenblatt, C.L. (1979). *Leishmania donovani*: Physiochemical, immunological and biological characterization of excreted factor from promastigotes. *Exp. Parasitol.* **47**, 254-269.

El-on, J., Zvillich, M., Sarov, I. (1990). *Leishmania major*: Inhibition of the chemiluminescent response of human polymorphonuclear leukocytes by promastigores and their excreted factors. *Parasite Immunol.* **12**, 285-195.

Eperon, S., McMahon-Pratt, D. (1989). I. Extracellular cultivation and morphological characterization of amastigote like forms of *Leishmania panamensis* and *L. braziliensis*. *J. Protozool*, **36**, 502-510.

Etges, R., Bouvier, J. and Bordier, C. (1986). The major surface protein of *Leishmania* promastigotes is a protease. *J. Biol. Chem.* **261**, 9098-9101.

Fan, X., Goldberg, M. and Bloom, B.R. (1988). Interferon-gamma induced transcriptional activation is mediated by protein kinase C. *Proc. Natl. Acad. Sci.* USA, **85**, 5122-5125.

Farah, F.S., Samra, S.A. and Nuwayri-Salti (1975). The role of the macrophage in cutaneous

leishmaniasis. *Immun.* 29, 755-764.

Farrell, J.P., Mueller, L and Louis, J.A. (1989). A role for Lyt-2$^+$ T-cells in resistance to cutaneous leishmaniasis in immunized mice. *J. Immun.* 142, 2052-2058.

Faster, F.N. (1962). Structure-activity relationship of amidine derivatives. *Pharm. Rev.* 14, 37.

Faust, E.C. and Russel, P.F. (1964). The blood and tissue flagellates. Protozoa and protozoan infections. Section II : in clinical parasitology edited by E.C. Faust and P.F. Russel, Elsevier, Amsterdam, pp. 104.

Ferguson, M.A.J., Brimacombe, J.S., Cottaz, S., Field, R.A., Guther, L.S., Homans, S.W., McConville, M.J. *et al.* (1994). Glycosyl-phosphatidylinositol molecules of the parasite and the host. *Parasitol.* 108, S45-S54.

Ferguson, M.A.J., Low, M.G., and Cross, G.A.M. (1985). Glycosyl-sn-1,2,-dimyristylphosphatidylinositol is covalently linked to *Trypanosoma brucei* variant surface glycoprotein. *J. Biol. Chem.* 260, 14547-555.

Ferguson, M.A.J. and Williams, A.F. (1988). Cell surface anchoring of proteins via glycosyl phosphatidyl inositol structures. *Annu. Rev. Biochem.* 57, 285-320.

Ferguson, M.A.J. (1991). Lipid anchors on membrane proteins. *Curr. Opin. Struct. Biol.* 1, 522-529.

Ferguson, M.A.J. (1992). Glycosylphosphatidylinositol membrane anchors: The tole of a tail. *Biochem. Soc. Trans.* 20, 243-256.

Finkelman, F.D., Madden, K.B., Cheever, A.W., Katona, I.M., Morris, S.C., Gately, M.K., Hubbard, B.R., Gauseand, W.C. and Urban, J.F. Jr. (1994). Effects of interleukin 12 on immune responses and host protection in mice infected with intestinal nematode parasites. *J. Exp. Med.* 179, 1563.

Flinn, H.M., Rangarajan, D. and Smith, D.F. (1994). Expression of a hydrophilic surface protein in infective stages of *L. major*. *Mol. Biochem. Parasitol.* 65, 259-270.

Fortier, A.H., Mock, B.A., Meltzer, M.S. and Nacy, C.A. (1987). *Mycobacterium bovis* BCG-induced protection against cutaneous and systemic *Leishmania major* infections in mice. *Infect. Immun.* 55, 1707-1714.

Franke, E.D., McGreevy, P.B., Katz, S.P., Sacks, D.L. (1985). Growth cycle dependent generation of complement resistant *Leishmania promastigotes*. *J. Immunol.* 134, 2713-2718.

Frankenburg, S., Leibovici, V., Mansbach, N., Turco, S.J., Rosen, G. (1990). Effect of glycolipids of *Leishmania* parasites on human monocyte activity inhbition by lipophosphoglycan. *J. Immunol.*

145, 4284-4289.

Frommel, T.O., Button, L.L., Fujikura, Y. and McMaster, W.R. (1990). The major surface glycoprotein (GP63) is present in both life stages of *Leishmnia. Mol. Biochem. Parasitol.* **38**, 25-32.

Fuzibet, J.G., Marty, P., Taillan, B., Bertrand, F., Pras, P., Pesce, A., Le Fichoux, Y., Dujarin, P. (1988). Is *Leishmania infantum* an opportunistic parasite in patients with anti-human immunodeficiency virus antibodies. *Arch. Intern. Med.* **148**, 1228.

Gajwani, F.B.W., Mehta, A., Sayed, B.A., Patil, N.N. and Pande, R.S. (1967). Kala-azar in Gujarat. *J. Indian Med. Assoc.* **49**, 216.

Garnham, P.C.C. and Humphrey, J.H. (1969). Problems in Leishmaniasis related to immunology. *Curr. Trop. Microbiol Immunol.* 20-42.

Ghalib, H.W., Whittle, J.A., Kubin, M., Hashim, F.A., El-Hassan, A.M., Grabstein, K.H., Trinchieri and Reed, S.G. (1995). IL-12 enhance Th1 Type responses in human *Leishmania donovani* infections. *J. Immunol.* **154**, 4623-4629.

Ghosh, A.K., Bhattacharya, F.K. and Ghosh, D.K. (1985). *Leishmania donovani*: Amastigote inhibition and mode of action of berberine. *Exp. Parasitol.* **60**, 404-413.

Giannini, M.S. (1974). Effects of promastigotes growth phase, frequency of subcultur, and host age on promastigote-initiated infections with *Leishmania donovani* in the golden hamsters. *J. Protozool.* **21**, 521-525.

Glaser, T.A., Baatz, J.E., Kreishman, G.P., Mukkada, A.J. (1988). pH homoestasis in *Leishmania donovani* amastigotes and promastigotes. *Proc. natl. Acad. Sci.* USA **85**: 7602-7606.

Glaser, T.A., Moody, S.F., Handman, E., Bacic, A. and Spithill, T.W. (1991). An antigenically distinct lipophosphoglycan on amastigotes of *Leishmania major. Mol. Biochem. Parasitol.* **45**:337-344.

Glevara, P., Alonso, G., Franco De Silveira, J., *et al* (1992). Identification of new world *Leishmania* using ribosomal gene spacer probe. *Mol. Biochem. Parasitol.* **56**, 15-26.

Glew, R.H., Saha, A.K. Das, S., Remaley, A.T. (1988). Biochemistry of *Leishmania* Parasites. *Microbiol Rev.* **52**, 412-432.

Goldstein, I.J. and Hayes, C. (1978). The lectins: Carbohydrate binding proteins of plants and animals. *Adv. Carbo. Chem. Biochem.* **35**, 127-340.

Gorezynski, R.M. (1985). Immunization of susceptible BALB/c mice against *Leishmania braziliensis*. II: Use of temperature-sensitive avirulent clones of parasite for vaccination purposes. *Cell. Immun.* **94**, 11-20.

Green, J.W. (1980). Oxidative reactions and degradations: In the carbohydrates ed. W. Pigman, 1B: 1101-1166. New York: Academic.

Green, S.I., Crawford, R.M., Hockmeyer, J.T., Meltzer, M.S., Nacy, C.A. (1990). *Leishmania major* amastigotes initiate the L-arginine-dependent killing machanism in IFN-γ-stimulated macrophages by induction of tumor necrosis factor-α. *J. Immunol.* **145**, 4290-4297.

Green, S.J., Nacy, C.A. and Meltzer, M.S. (1991). Cytokine induce synthesis of nitrogen oxides in macrophages: A protective host response to *Leishmania* and other intra-cellular pathogens. *J. Leuko. Biol.* **50**, 93-103.

Greenblatt, C.L., Slutzky, G.M., de Ibarra, A.A.L., Snary, D. (1983). Monoclonal antibodies for serotyping of *Leishmania strains*. *J. Clin. Microbiol.* **18**, 191-193.

Greis, K.D., Turco, S.J., Thomas, J.R., McConville, J.J., Homans, S.W., Ferguson, M.A.J. (1992). Purification and characterization of an extracellular phosphoglycan from *Leishmania donovani*. *J. Biol. Chem.* **267**, 5876-5881.

Grimaldi, G., Moriearty, P.L. and Hoff, A. (1980). *Leishmania mexicana* in C3H mice-BCG and levanisole treatment of established lesion. *Clin. and Exp. Immun.* **41**, 237-242.

Grogl, M., Oduola, A.M.J., lawrence, D.C.C. and Kay, D.E. (1989). *Leishmania* spp.: Development of pentostam-resistant clones *in vitro* by disconuous drug exposure. *Exp. Parasitol.* **69**, 78-90.

Grun, J.L. and Maurer, R.H. (1989). Different T helper cell subsets elicited in mice utilising two different adjuvant vehicles the role of endogenous IL-1 in proliferative resposes. *Cell. Immunol.* **121**, 134-145.

Gueugnot, J., Guillot, J., Damez, M. and Coulet, M., (1984). Identification and taxonomy of human and animal *Leishmania* by lectin-mediated agglutination. *Acta Tropica* **41**: 135-143.

Guilian, G.G., Moss, R.L. and Greaser, M. (1983). Improved methodology for analysis and quantitation of proteins on one dimensional silver stained slab gels. *Anal. Biochem.* **129**, 277-287.

Guilian G.G., Shanahan, M.F., Graham, J.M. and Moss, R.L. (1985). Resolution of low molecular weight polypeptides in a non-urea sodium dodecyl sufate slab polyacrylamide gel system. *Fed. Proc.* **44**, 686.

Gupta, S. and Bhatia, R.K. (1975). India Practitioner, **28**, 609.

Guy, R.A. and Belosevic, M. (1993). Comparison of receptors required for entry of *Leishmania major* amastigotes into macrophages. *Infect. Immun.* **61**, 1553-1558.

Hamilton, T.A., and Adams, D.O. (1987). Molecular mechanisms of signal transduction in

macrophages. *Immunol. Today* 8, 151.

Handman, E (1990). Study of *Leishmania major*-infected macrophages by use of lipophosphoglycan-specific monoclonal antibodies. *Infect. Immun.* 58, 2297-2302.

Handman, E., Goding J.W. (1985). The *Leishmania* receptors for macrophage is a lipid containing glycoconjugate. *EMBO J.* 4, 329-336.

Handman, E., Greenblatt, C.L. and Goding, J.W. (1984). An amphipathic sulphated glycoconjugate of *Leishmania* characterised with monoclonal antibodies. *EMBO J.* 3, 1206-1210.

Handman, E., Greenblatt, C.L. (1977). Promotion of leishmanial infections in non-permissive host macrophages by conditioned medium. *Z. Parasitenkd.* 53, 143-147.

Handman, E., Mitchell, G.F. (1985). Immunization with *Leishmania* receptors for macrophages protects mice against cutaneous leishmaniasis. *Proc. Natl. Acad. Sci. USA*, 82, 5910-5914.

Handman, E., Schnur, L.F., Spithill, T.W., Mitchell, G.F. (1986). Passive transfer of *Leishmania* lipopolysaccharide confers parasite survival in macrophages. *J. Immunol.* 137, 3608-3613.

Handman, E. and Hocking, R.E. (1982). Stage specific strain specific and crossreactive antigens of *Leishmania* species identified by monoclonal antibodies. *Infect. Immu.* 37, 28-33.

Hanson, B.A. and Lester, R.L. (1980). The extraction of inositol-containing phospholipids and phosphotidylcholine from *Saccharomyces cerevisiae and Neurospora crassa. J. Lipid Res.* 21, 309-315.

Hart, D.T., Opperdoes, F.R. (1984). The occurrence of glycosomes (micro bodies) in the promastigote stage of four major *Leishmania* species. *Mol. Biochem. Parasitol.* 13, 159-172.

Hassan, M. Q., Ghosh, A., Ghosh, S.S., Gupta, M., Basu, D., *et al* (1993). Enzymatic amplification of mini-exon derived RNA gene spacers of *Leishmania mexicana* primers and probes for DNA diagnosis. *Parasitol.* 44, 63-72.

Heinzel, F.P., Schoenhaut, D.S., Rerko, R.M., Roser, L.E. and Gately, M.K. (1993). Recombinant IL-12 cures mice infected with *Leishmania major. J. Exp. Med.* 177, 1505-1510.

Heinzel, F.P. Sadick, M.D., Holaday, B.J. Coffman, R.L. and Locksley, R.M. (1989). Receiprocal expression of interferon-γ or interleukin 4 during the resolution or progression of murine leishmaniasis. Evidence for expansion of distinct helper T-cell subsets. *J. Exp. Med.* 169, 59-72.

Hendricks, L.D., Wright, N. (1979). Diagnosis of cutaneous Leishmaniasis by *in vitro* cultivation of saline aspirates in schneider's Drosophila medium *Am. J. Trop. Med. Hyg.*, 28, 962.

Hernandez, A.G. (1982). Lectins as a tool in parasite research. *In* Biochemical characterization of

Leishmania M.L. Chance and B.C. Walton (eds). UNDP/World Bank/WHO. Geneva. pp 181-196.

Hernendez, A.G., Arguello, C. Ayesta, C., *et al.* (1981). The surface membrane of *Leishmania* P-47-65 in G.M. Slutzky (ed). The biochemistry of parasites. Pergamom Press. Inc. Elmsford, N.Y.

Herwaldt, B.L. and Berman, J. (1992). Recommendations for treating leishmaniasis with sodium stibogluconate (pentostam) and review of pertinent clinical studies. *Am. J. Trop. Med. Hyg.* **46**, 296-302.

Heumann, D., Burger, D., Vischer, t., De Colmenares, M., Bouvier, J. & Bordier, C. (1989). Molecular interactions of *Leishmania* promastigote surface protease with humanα2-macroglobulin. *Mol. Biochem. Parasitol.* **33**, 67-72.

Heyneman, D. (1963). Leishmaniasis in the Sudan Republic. Comparison of experimental *Leishmania donovani* infections in *Phlebotomus papatasi* with natural infections found in man-baited *P. orientalis* captured in a kala-azar endemic region of the sudan, *Proc. Roy. Soc. London.* **B199**, 309-320.

Hibbs, J.B.Jr., Taintor, R.R., Vavrin, Z. and Rachlin, E.M. (1988). Nitric oxide: A cytotoxic activated macrophage effector molecule. *Biochem. Biophys. Res. Comm.* **157**, 87-94.

Hill, J.O., Awwad, M. and North, R.J. (1989). Elimination of CD4 suppressor T cells from susceptible BALB/c mice releases CD8 T lymphocytes to mediate protective immunity against *Leishmania. J. Exp. med.* **169**, 1819-1827.

Hock, H., Dorsch, M. *et al* (1991). Interleukin 7 induces CD4+ T cell dependent tumor rejection. *J. Exp. Med.* **174**, 1291-1299.

Hockmeyer, W.T., Kager, P.A., Rees, P.H. Hendricks, L.D. (1981). The culture of *L. donovani* in schneider's insect medium. Its value in the diagnosis and management of patients with visceral leishmaniasis. *Trans. Roy. Soc. Trop. Med. Hyg.* **75**, 861-863.

Hoffman, D.R., Hadju, J., Snyder, F. (1984). Cytotoxicity of platelet-activating factor related alkyl-phospholipid analogs in human leukemia cells, polymorphonuclear neutrophils, and skin fibroblasts. *Blood* **63**, 545-552.

Homans, S.W., Melhert, A., Turco, S.J. (1992). Solution structure of the lipophosphoglycan of *Leishmania donovani. Biochem.* **31**, 654-661.

Hoover, D.L., Berger, M., Hammer, C.H. and Meltzer, M.S. (1985). Complement mediated serum cytotoxicity for *Leishmania major* amastigotes: killing by serum deficient in early components of the membrane attack complex. *J. Immun.* **135**, 570-574.

Howard, M.K., Sayers, G. and Miles, M.A. (1987) *Leishmania donovani* metacyclic promastigotes:transformation *in vitro*, lectin, agglutination, complement resistance, and infectivity.

Exp. Parasitol. **64**, 147-156.

Huang, C. and Turco, S.J. (1993). Defective galactofuranose addition in lipophosphoglycan biosynthesis in a mutant of *Leishmania donovani*. *J. Biol. Chem.* **268**, 24060-24066.

Hunter, K.W., Cook, C.L., Hayunga, E.G. (1984). *Leishmania* differentiation *in vitro* induction of heat shock proteins. *Biochem. Biophys. Res. Commun*, **125**, 755-760.

Ilg, T., Etges, R., Overath, P., McConville, M.J. Thomas, J.R. *et al.* (1992). Structure of *Leishmania mexicana* lipophophoglycan. *J. Biol. Chem.* **267**, 6834-6840.

Ilg, T., Harbecke, D. and Overath, P. (1993). The lysosomal gp63-related protein in *Leishmania mexicana* amastigotes is a soluble metalloproteinase with an acidic pH optimum. *Feb. Eur. Biochem. Soc.* **327**, 103-107.

Ilg, T., Menz, B., Winter, G., Russell, D.G., Etges, R., *et al.* (1991). Monoclonal antibodies to *Leishmania mexicana* promastigote antigens. Secreted acid phosphatase and other proteins share epitopes with lipophosphoglycan. *J. Cell Sci.* **99**, 175-180.

Ilg, T., Stierhof, Y.D., Wiese, M., McConville, M.J. and Overath, P. (1994) Characterization of phosphoglycan-containing secretory products of *Leishmania*. *Parasitol.* **108**, S63-S71.

Iyer, R.N. (1985). Current trends in the chemotherapy of leishmanaisis. In: Perspectives in parasitology edited by A.B. Sen, J.C. Katiyar and P.Y. Guru. Print House, India, **1**, 275.

Jackson, J.E.D., Honigberg, B.M. (1978). Glycoproteins relationships with host and bacterial antigen and preliminary biochemical analysis. *J. Protozool.* **25**, 514-525.

Jacob, V.P. and Kalra, S.L. (1951). Kala-azar in Kashmir. *Ind. J. Med. Res.* **39**, 329.

Jacobson, R.L., and Schnur, L.F. (1987). Variation in surface membrane carbohydrates of *Leishmania major* strains classified by enzyme profiles. 3rd Mediterrancan Conference of Parasitology. Jerusalem, p. 93.

Jacobson, R.L. and Doyle, R.L. (1996). Lectin-Parasite interactions. *Parasitol. Today* **12**, 55-61.

Jacobson, R.L. (1995) *Leishmania*, LPG and the sandfly connection. *Parasitol Today*, **11**, 203-204.

Jaffe, C.L., Martinez, L.P., Sarfstein, R. (1989). *Leishmania tropica*: characterization of lipophosphoglycan-like antigen recognized by species specific monoclonal antibodies. *Exp. Parasitol.* **70**, 12-24.

Jaffe, C.L., Perez, L., Schnur, L.F. (1990). Lipophosphoglycan and secreted acid phosphatase of *Leishmania tropica* share species specific epitopes. *Mol. Biochem. Parasitol.* **41**, 233-240.

Jaffe, C.L. and McMahon-Pratt, D. (1988). The identification of membrane glycoconjugate in *Leishmania* species. *J. Parasitol.* 74, 548-561.

James, S.L. and Hibbs, J.B. (1990). The role of nitrogen oxides as effector molecules of parasite killing. *Parasitol. Today* 5, 303-305.

Jardim, A., Alexander, J. Teh, H.S., Ou, D. and Olafson, R.W. (1990). Immunoprotective *Leishmania major* synthetic T cell epitopes. *J. exp. med.* 172, 645-648.

Jardim, A., Hanson, S. Ullman, B., MCubbin, W.D. *et al.* (1995). Cloning and structure-function analysis of the *Leishmania donovani* kinetoplastid membrane protein II *Biochemical J.* 305, 315-320.

Jardim, A., Tolson, D.L., Turco, S.J., pearson, T.W., Olafson, R.W. (1991). The *Leishmania donovani* lipophosphoglycan T-lymphocyte reactive component is a tightly associated protein complex. *J. Immunol.* 147, 3538-3544.

Jayaraman, K.S. (1988). Leishmaniasis resurgent in India. *Nature* (London) 333, 590.

Jha, T.K. (1983). Evaluation of diamidine compound (Pentamidine isethionate) in the treatment of resistant cases of Kala-azar occuring in North Bihar, India. *Trans. Roy. Soc. Trop. Med. Hyg.* 77, 163.

Joiner, K. A. (1988). Complement evasion by bacteria and parasites. *Annu. Rev. Microbiol.* 42, 201-230.

Kahl, L.P., Lelchuk, R., Scoot, C.A. and Beesley J. (1990). Characterization of *Leishmania major* antigen-liposomes that protect BALB/c mice against cutaneous leishmaniasis. *infect. Immun.* 58, 3233-3241.

Kain, K.C., Orlandi, P.A. Haynes, J.D., Sin, B.K.L. and Lanar, D.E. (1993). Evidence for two stage binding by the 175 kD erythocyte binding antigen of *Plasmodium falciparum*. *J. Exp. Med.* 178:1497-1515.

Kapur, M.R., Chopra, S.K. and Hasan, M. (1979). *Ind. J. Pathological Microbial.* 22, 85. Cited from Indo-UK Workshop on *Leishmaniasis*. *ICMR*, New Delhi (1983).

Karen, L. Goa and Deborah, M., Compoli-Richards (1987). Pentamidine isethionate: A review of its antiprotozoal activity, Pharmacokinetics, properties and therapeutic use in *Pneumocystic carinii*. *Pneumonia Drug.* 33, 248.

Karp, C.L., Turco, S.J., Sacks, D.L. (1991). Lipophospho-glycan masks recognition of the *Leishmania donovani* promastigote surface by human Kala-azar serum. *J. Immunol*, 147, 680-684.

Kaufmann, S.H.E. (1988). $CD8^+$ T lymphocytes in intracellular microbical infections. *Immunol. Today* **8**, 168-174.

Kaye, P.M., Curry, A.J. and Blackwell, J.M. (1991). Differential production of Th1 and Th2 cytokines does not determine the genetically controlled or vaccine induced rate of cure in murine visceral leishmaniasis. *J. Immunol.* **146**, 2763-2770.

Kaye, P.M., Curry, A.J. Bancroft, G.J. and Lang, T. (1991). Antigen Processing and Presentation modelling with *Leishmania. Behr. Mittel. Inst.* **88**, 13-19.

Kaye, P.M., Gorak, P., Murphy, M. and Ross, S. (1995). Strategies for immune intervention in visceral leishmaniasis. *Ann. Trop. Med. Parasitol.* **89**, 75-81.

Kaye, P.M. (1987). Antigen presentation and the response to parasitic infection. *Parasitol. Today* **3**, 293-299.

Keegan, F.P.L., Sanzone, L., Blun, J.J. (1987). Oxidation of glucose, ribose, alanite and glutamate by *Leishmania braziliensis panamensis. J. Protozool.* **34**, 174-179.

Kelleher, M., Curtis, J.M., Sacks, D.L., Handman, E. and Bacic, A. (1994) Epitope mapping of monoclonal antibodies directed against Lipophosphoglycan of *Leishmania major* promastigotes. *Mol. Biochem. Parasitol.* **66**, 187-200.

Kelleher, M., Moody, S.F., Mirabile, P., Osborn, A.H., Bacic, A. and Handman, E. (1995). Lipophosphoglycan blocks attachment of *Leishmania major* amastigotes to macrophages. *Infect. Immun.* **63**, 43-50.

Kelleher, M. Bacic, A. and Handman, E. (1992) Identification of a marophage-binding determinant on lipophosphoglycan from *Leishmania major. Proc. Natl. Acad. Sci.* USA, **89**, 6-10

Kemp, M., Theander, T.G. and Kharazmi, A. (1996). The contrasting role of CD_4^+ T-cells in intracellular infections in humans: Leishmaniasis as an example. *Immunol. Today* **17**, 13-16.

Khalid, F., Bennaceus, B., Benothman, H., Achouri, E., Ayachi, R. and Regaieq, R. (1990). Severe liver involvement in the course of Kala-azar, seven case report. *Archives Francaises de Pediatrico* **47(4)**, 257.

Killick-Kendrick, R., Molyneux, D.H., Hommel, M., Leaney, A.J. and Robertson, E.S. (1977). *Leishmania* in phlebotomid sandflies. V. The nature and significance of infections of the pylorus and ileum of the sandfly by Leishmanial of the Braziliensis complex. *Proc. Roy Soc. London.* **198**, 191-199.

Kimsey, P.B., Theodos, C.M., Mitchen, T.K., Turco, S.J. and Titus, R.G. (1993). An avirulent lipophosphoglycan-deficient *leishmania major* clone induces $CD4^+$ T cells which protect susceptible BALB/c mice against infection with virulent *L. major. Infect. Immun.* **61**, 5205-5213.

King, D.L., Chang, Y.D., Turco, S.J. (1987). cell surface lipophosphoglycan of *Leishmania donovani*. *Mol. Biochem. Parasitol.* **24**, 47-53.

King, D.L., Turco, S.J. (1988). A ricin agglutinin-resistant clone of *Leishmania donovani*. *Mol. Biochem·Parasitol.* **28**, 285-294.

Korn, E.D. and Wright, P.L. (1973). Macromolecular composition of an Amoeba plasma membrane. *J. Biol. Chem.*. **248**, 439-447.

Kreutzer, R.D. and Christensen, H.A. (1980). Characterization of *Leishmania species* by isoenzyme electrophoresis. *Am. J. Trop. Med. Hyg.* **29**, 199-208.

Kubin, M., Chow, J.M. and Trinchieri, G. (1994). Differential regulation of interleukin-12, tumor necrosis factor-α and interleukin-1β production in human myeloid leukemia cell lines and peripheral blood mononuclear cells. *Blood* **83**, 1847.

Kurtzhals, J.A.L., Hey, A.S., Jardim, A. *et al.* (1994). Dichotomy of the human T cell response to *Leishmania* antigens. II absent or Th2-like response to gp63 and Th-1 like response to LPG associated protein in cells from cured visceral leishmaniasis patients. *Clin. Exp. Immunol.* **46**, 416-421.

Kurtzhals, J.A.L., Hasen, M.B., Hey, A.S. and Poulsen, L.K. (1992). Cellular and humoral immune responses in a population from the Baringo district, Kenya to *Leishmania* promastigote lipophosphoglycan. *J. Immunol. Methods* **156**, 239-245.

Kurt-Jones, E.A., Hamberg, S., Ohara, J., Paul, W.E. and Abbas, A.K. (1987). Heterogeneity of helper/inducer T lymphocytes. 1: Lymphokine production and lymphokine responsiveness. *J. Exp. Med.* **166**, 1774-1787.

Lainson, R., Shaw, J.J. (1972). Leishmaniasis of the new word: taxonomic problems. *Br. Med. Bull.* **28**, 44-48.

Lang, T., Warburg, A., Sacks, D.L., Croft, S., Lane, R.P. and Blackwell, J.M. (1991). Transmission and scanning EM immunogold labelling of *Leishmania major* lipophosphoglycan in the sandfly *Phlebotomus papatasi*. *Eur. J. Cell Biol.* **55**, 362-372.

Laskay, T., Gemetchu, T., Teferedegn, H. and Frommel, D. (1991). The use of DNA for detection of *Leishmania aethiopica* in naturally infected sandfly vectors. *Trans. Roy. Soc. Trop. Med. Hyg.* **85**, 599-602.

Laskey, L.A. (1992). Selections: interpreters of cell-specific carbohydrate information during inflammation. *Science.* **258**, 964-969.

Leon, L.L., Soares, M.J. and Temporal, R.M. (1995). Effects of temparature on promastigotes of several species of *Leishmaia*. *J. Euk. Microbiol.* **42**, 219-223.

Lester, R.L., Smith, S.W., Wells, G.B., Rees, D.C. and Angus, W.W. (1974). The isolation and partial characterization of two novel sphingolipids from *Neurospora crassa*: Di (Inositol phosphoryl)ceramide and [(gal)3glu]ceramide. *J. Biol. Chem.* **249**, 3388-3394.

Lewis, D.H. and Peters, W. (1977). The resistance of intracellular *Leishmania* parasites to digestion by lysosomal enzymes. *Ann. Trop. Med. Parasitol.* **71**, 295-312.

Liew, F.Y., Millott, S., Li, Y., Lelchuk, R., Ling Chan, W. and Ziltener, H. (1989). Macrophage activation by interferon-γ from host-protective T cells in inhibited by interleukin (IL)3 and IL-4 produced by disease promoting T cells in leishmaniasis *Eur. J. Immunol.* **19**, 1227-1232.

Liew, F.Y., Singleton, A. Ciliari, E. and Howard, J.G. (1985b). Prophylactic immunization against experimental leishmaniasis. V. Mechanism of the antiprotective blocking effect induced by subscutaneous immunization against *Leishmania major*. *J. Immunol.* **135**, 2101-2107.

Liew, F.Y., Hale, C. and Howard, J.G. (1982). Immunological regulation of experimental cutaneous leishmaniasis. V: Characterization of effector and specific suppressor T cells. *J. Immunol.* **128**, 1917-1922.

Liew, F.Y., Li, Y. and Millott, S. (1990a). Tumour necrosis factor-α-synergises with IFN-γ in mediating killing of *Leishmania major* through the induction of nitric oxide. *J. Immunol.* **145**, 4306-4310.

Liew, F.Y., Li, Y. and Millott, S. (1990b). Tumour necrosis factor (TNEα) in leishmaniasis II. TNF α-induced macrophage leishmanicidal activity is mediated by nitric oxide from L-arginine. *Immunol.* **71**, 556-559.

Liew, F.Y., Parkinson, C. Millott, S., Severn, A. *et al* (1990). Tumor necrosis factor in leishmaniasis I. TNFα mediates host protection against cutaneous *leishmaniasis*. *Immunol.* **69**, 570-3.

Liew, F.Y., Stephanie, M., Mittot and Jerzy, A. Schmidt. (1990). A repetitive peptide of *Leishmania* can activate 'T' helper type 2 cells and enhance disease progression. *J. Exp. Med.* **172**, 1359.

Liew, F.Y. and Cox, F.E.G. (1991). Nonspecific defence mechanism: the role of nitric oxide. *Immunoparasitol. Today* **A17**, 21.

Liew, F.Y. and O'Donnell, C.A. (1993). Immunology of leishmaniasis. *Adv. Parasitol.* **32**, 160-259.
Liew, F.Y. (1989). Functional heterogeneity of CD4+ T-cells in leishmaniasis. *Immunol. Today* **10**, 40-45.

Liew, F.Y. (1990). Regulation of cell-mediated immunity in leishmaniasis. *Cur. Top. Microbiol. Immunol.* **55**, 53-64.

Ling, M., Stafford, A., Nir, S., Turco, S.J., Flanagan, T.D. and Epand, R.M. (1995). Potent inhibition of viral fusion by the lipophosphoglycan of *Leishmania donovani*. *Biochem.* **34**, 4676-

4683.

Llanos-cuentas, A. *et al.* (1991). *Kidney Int.* **40**, 302-308.

Locksley, R.M., Heinzel, F.P., Holaday, B.J., Mutha, S.S., Reiner, S.L. and Sadick, M.D. (1991). Induction of Th_1 and Th_2 CD4+ subsets during murine *Leishmania major* infection. *Res. Immunol.* **142**, 28-32.

Lopez, A.J., Reins, H.A., Etges, R.J., Button, L.L., McMaster, W.R., Overath, P. and Klein, J. (1991). Genetic control of the immune response in mice to *Leishmania mexicana* surface protease. *J. Immunol.* **146**, 1328-1334.

Lopez, M., Inga, R., Cangalaya, M., *et al* (1993). Diagnosis of *Leishmania* using the polymerase chain reaction: a simplified procedure for field work. *Am. J. Trop. Med. Hyg.* **49**, 348-356.

Louis, J.A., Zubler, r.H., Coutinho, S.G., Lima, G., *et al* (1982). The *in vitro* generation and functional analysis of murine T cell populations and clones specific for a protozoan parasite. *Leishmania tropica Immunol. Rev.* **61**, 215-243.

Love, D.A., Esko, J.D. and Mosser, D.A. (1993). A heparin-binding activity on *Leishmania* amastigotes which mediates adhesion to cellular proteoglycans. *J. Cell. Biol.* **123**, 759-766.

Low, M.G. (1989). The glycosyl-phosphatidyl inositol anchor of membrane proteins. *Biochim. Biophys. Acta.* **988**, 427-454.

Lowry, D.H., rosebrough, N.J., Farr, A.L. and Randal, R.J. (1951). Protein measurement with the Folin-phenol reagent. *J. Biol. Chem.* **193**, 265-275.

Maingon, R., LeRay, D. and Hommel, M. (1995) Leishmaniasis research and the european union. *Parasitol. Today* **11**, 357-359.

Mallinson, D.J. and Coombs, G.H. (1989). Interaction of *Leishmania* metacyclics with macrophages. *Int. J. Parasitol.* **19**, 647-656.

Manson-Bahr, P.E.C. (1961). Immunity in Kala-azar. *Trans. Roy. Soc. Trop. Med. Hyg.* **55**, 550-555.

Marletta, M.A., Yoon, P.S., Iyengar, R., Leaf, C.D. and Wishnok, J.S. (1988). Macrophage oxidation of L-Arginine to nitrite and nitrate: Nitric oxide is an intermediate. *Biochem.* **27**, 8706.

Marr, J.J., Berens, R.L. and Nelson, D.J. (1978). Purine metabolism in *L. donovani* and *L. brasiliensis*. *J. Protozool.* **25**, 15A.

Mauel, J., Ransijn, A. and Bachmuller-Rouillev, Y. (1991). Killing of *Lieshmania* parasites in activated murine macrophages is based on an L-arginine dependent process that produces nitrogen

derivatives. *J. Leuk. Biol.* 49, 73-82.

McConville, M., Turco, S.J., Ferguson, M. and Sacks, D.L. (1992). Developmental modification of lipophosphoglycan during the differentiation of *Leishmania major* promastigotes to an infectious stage. *EMBO J.* 11, 3593-3600.

McConville, M.J., Bacic, A., Mitchell, G.F., Handman, E. (1987). Lipophosphoglycan of *Leishmania major* that vaccinates against cutaneous leishmaniasis contains an alkylglycero phosphatidylinositol lipid anchor. *Proc. Natl. Acad. Sci.* USA 84, 9841-9845.

McConville, M.J., Bacic, A. (1989). A family of glycoinositol phospholipids from *Leishmaia major*. *J. Biol. Chem.* 264, 757-766.

McConville, M.J., Blackwell, J.M. (1991). Developmental changes in the glycosylated phosphatidyl inositols of *Leishmania donovani J. Biol. Chem.* 266, 15170-15179.

McConville, M.J., Collidge, T., Ferguson, M.A.J. and Schneider, P. (1993). The glycoinositol phospholipids of *L. mexicana* promastigotes- Evidence for the presence of three distinct pathways of glycolipid biosynthesis. *J. Biol. Chem.* 268, 15595-15604.

McConville, M.J., Homans, S.W., Thomas-Oates, J.E., Dell, A. and Bacic, A. (1990). Structures of the glycoinositolphospholipids from *Leishmania major* a family of novel galactofuranose-containing glycolipids. *J. Biol. Chem.* 265, 7385-7394.

McConville, M.J., Schnur, L.F., Jaffe, C. and Schneider, P. (1995). Structure of *Leishmania* lipophosphoglycan: inter and intra-specific polymorphism in Old World species. *Biochem. J.* 310, 807-818.

McConville, M.J. and Bacic, A. (1990). The glycoinositolphospholipids profiles of two *L. major* strains that differ in lipophosphoglycan expression. *Mol. Biochem. Parasitol.* 38, 57-68.

McConville, M.J. and Ferguson, M.A.J. (1993). The structure, biosynthesis and function of glycosylated phosphatidyl inositols in the parasitic protozoa and higher eukaryotes. *Biochem. J.* 294, 305-324.

McConville, M.J. and Homans, S.W. (1992). Identification of the defect in lipophosphoglycan biosynthesis in a nonpathogenic strain of *Leishmania major*. *J. Biol. Chem.* 267, 5855-5861.

McConville, M.J. Thomas-Oates, J.E., Ferguson, M.A.J., Homans, S.W. (1990). Structure of the lipophosphoglycan from *Leishmania-major*. *J. Biol. Chem.* 265, 19611-19621.

McConville, M.J. (1991). Glycosylated phosphatidylinositols as virulence factors in *Leishmania Cell. Biol. Int. Rep.* 15, 779-798.

McElrath, M.J., Murray, H.W. and Cohn, Z.A. (1988). The dynamics of granuloma formation in

experimental visceral leishmaniasis. *J. Exp. Med.*. **167**, 1927-1937.

McGruvy, P.B. and Marsden, P.D. (1986). Chemotherapy of parasitic diseases Eds. Campbell, W.C. and Rew, R.S.; Plenum Press, New York, 123.

McMahon-Pratt, D., Rodriglez, D., Rodriguez, J.R. *et al* (1993). Recombinant vaccinia viruses expressing GP42/M-2 protect against *L. infection. Infect. Immun.* **61**, 3351-3359.

McMahon-Pratt, D. and David, J.R. (1982). Monoclonal antibodies that distinguish between New World species of *Leishmania. Nature* (London) **291**: 581-583.

McNeely, T.B., Rosen, G., Londner, M.V., Turco, S.J. (1989). Inhibitory effects on protein kinase C by lipophosphoglycan and glycosyl phosphatidylinositol antigens of *Leishmania. Biochem. J.* 259, 601-604.

McNeely, T.B., Tolson, D.L., Pearson, T.W. and Turco, S.J. (1990). Characterization of *Leishmania donovani* variant clones using anti-lipophosphoglycan monoclonal antibodies. *Glycobiol.* **1**, 63-69.

McNeely, T.B., Turco, S.J. (1987). Inhibition of protein kinase C by the *Leishmania donovani* Lipophosphoglycan *Biochem. Biophys. Res. Commun.* **148**, 653-657.

McNeely, T.B., Turco, S.J. (1990). Requirement of lipophosphoglycan for intracellular survival of *Leishmania donovani* with in human monocytes *J. Immunol.* **144**, 2754-2750.

Medina-Acosta, E., Karess, R.E., Schwartz, H. and Russell, D.G. (1989). *Mol. Biochem. Parasitol.* **38**, 263-273.

Medina-Acosta, E., Karess, R.E. and Russell, D. (1993). Structurally distinct genes for the surface protease (gp63) of *Leishmania mexicana* are developmentally regulated. *Mol. Biochem. Parasitol.* **57**, 31-46.

Medina-Acosta, E.I., Beverley, S.M. and Russell, D.G. (1993). Evolution and expression of the *Leishmania* surface proteinase (gp63) gene locus. *Infect. Agent. Dis.*, **2**, 25-34.

Mehta, K., Juliano, R.L., Lopez-Berestein, G. (1985). Stimulation of macrophage protease secretion via liposomal delivery of muramyl dipeptide derivatives to intracellular sites. *Immunol.* **51**, 517-527.

Mehta, K., Lopez-Berestein, G., Hersh, E.M., Juliano, R.L. (1982). Uptake of liposome and liposome encapsulated muramyl dipeptide by human monocytes. *J. Reticuloendo. Soc.* **32**, 155-164.

Mendonca, S.C.F., Russell, D.G., Coutinho, S.G. (1991). Analysis of the human T-cell responsiveness to purified antigens of *Leishmania:* Lipophosphoglycan (LPG) and glycoprotein 63 (gp63). *Clin. Exp. Immunol.* **83**, 472-478.

Menon, A.K., Schwarz, R.T., Mayor, S. and Cross, G.A.M. (1990). Biosynthesis of glycosylphosphatidylinositol lipids in *Trypanosoma brucei*: involvement of mannosylphosphoryldolichol as the mannose donor. *J. Biol. Chem.* **265**, 9033-9042.

Miller, H.C. and Twonhy, D.W. (1967). Infection of macrophages in culture by leptomonads of *L. donovani*. *J. Protozool.* **14**, 781.

Milon, G., Giudice, G.D. and Louis, J.A. (1995). Immunology of experimental cutaneous leishmaniasis. *Parasitol. Today* **11**, 244-247.

Mishra, M., Biswas, U.K., Jha, A.M. and Khan, A.B. (1994). Amphotericin versus sodium stibogluconate in first line treatment of Indian kala-azar. *Lancet.* ii, 1599-1600.

Mishra, M., Biswas, U.K., Jha, D.N., Khan, A.B. (1992). Amphotericin versus pentamidine in antimony-unresponsive kala-azar. *Lancet.* **340**, 1256-1257.

Mitchell, G.F., Curtis, J.M., Scollay, R.G. and Handman, E. (1981). Resistance and abrogation of resistance to cutaneous leishmaniasis in reconstituted BALB/c nude mice. *Aus. J. Exp. Bio. Med. Sci.* **59**, 539-554.

Modabber, F. (1989). Experiences with vaccines against cutaneous leishmaniasis: of man and mice. *Parasitol.* **88** (suppl.) S49-S60.

Modabber, F. (1995). Vaccines against leishmaniasis. *Ann. Trop. Med. Parasitol.* **89**, 83-88.

Moll, H., Mitchell, G.F., McConville, M.J., Handman, E. (1989). Evidence for T-cell recognition of mice of a purified lipophosphoglycan from *L. major*. *Infect. Immun.* **57**, 3349-3356.

Moody, S.F., Handman, E., Bacic, A. (1991). Structure and antigenicity of the Lipophosphoglycan from *Leishmania major* amastigotes. *Glycobiol.* **1**, 419-424.

Moody, S.F., Handman, E., McConville, M.J. and Bacic, A. (1993). The structure of *Leishmania major* amastigote lipophosphoglycan. *J. Biol. Chem.* **268**, 18457-18466.

Moore, K.W., Vieira, P., Fiorenitino, D.F,, Trounstine, M.L., Khan, T.A. and Mosmann, T.R. (1990). Homology of cytokine synthesis inhibitor factor (IL-10) to the Epstein-Barr virus gene BCRFI. *Sic.* **248**, 1230.

Mosmann, I.R., Cherwinski, H., Bond, M.W., Giedlin, M.A. and Coffman, R.L. (1986). Two types of murine helper T cell clone. I: Definition according to profiles of lymphokine activities and secreted proteins. *J. Immunol.* **136**, 2348-2357.

Mosser, D.M., Edelson, P.J. (1984). Activation of the alternative complement pathway by *Leishmania* promastigotes: Parasite lysis and attachment to macrophages. *J. Immunol.* **132**, 1501-1505.

Mosser, D.M., Edelson, P.J. (1985). The mouse macrophages receptor for C3bi (CR3) is a major mechnaism in the phagocytosis of *Leishmania* promastigotes.*J. Immunol.* **135**, 2785-2789.

Mosser, D.M., Springer, T.A. and Diamond, M.S. (1992). *Leishmania* promastigotes require opsonic complement to bind to the human leukocyte integrin Mac-1 (DC11/CD18). *J. Cell Biol.* **116**, 511-520.

Mosser, D.M. Edelson, P.J. (1987). The third compoent of complement (C_3) is responsible for the intracellular survival of *Leishmania major. Nature* **327**, 329-331.

Mougneau, E., Altare, F., Wakil, A.E., Zheng, S., Coppola, T., Wang, Z., Waldman, R., Loicksley, R. and Glaichenhaus, N. (1994). Expression cloning of a *Leishmania major* protective T Cell antigen. *Science.* **268**, 563-566.

Mukherjee, S., Ghosh, C. and Basu, M.K. (1988). *Leishmania donovani*: Role of microviscosity of macrophage membrane in the process of parasite attachment and internalization. *Exp. Parasitol.* **66**, 18-26.

Muller, I. and Louis, J.A. (1989). Immunity to experimental infection with *Leishmania major.* Generation of protective L3T4+ T cell clones recognizing antigen(s) associated with live parasites. *Eur. J. Immunol.* **19**, 865-871.

Murray, H.W., Berman, J.D. and Wright, S.D. (1988). Immunochemotherapy for intracellular *Leishmania donovani* infection: γ-interferon plus pentavalent antimony. *J. Infect. Dis.* **157**, 973-978.

Murray, H.W., Carriero, S.M. and Donelly, D.M. (1986). Presence of a macrophage mediated suppressor cell mechanism during cell-mediated immune response in experimental visceral leishmaniasis.*Infect. Immun.* **54**, 487-493.

Murray, H.W., Cervia, J.S., Hariprashad, J., Taylor, A.P., Stoeckle, M.Y., Hockman, H. (1995). Effect of granulocyte macrophage colony-stimulating factor in experimental visceral leishmaniasis. *J. Clin. Invest.* (in press).

Murray, H.W., Miralles G.D., Stoeckle, M.Y., McDermott, D.F. (1993). Role and effect of interleukin-2 in experimental visceral leishmaniasis. *J. Immunol.* **151**, 929-934.

Murray, H.W., Stern, J.J., Welte, K., Rubin, B.Y., Carriero, S.H. and Nathan, C.F. (1987). Experimental visceral leishmaniasis: production of interleukin 2 and interferon-γ.*J. Immunol.* **138**, 2290-2297.

Murray, H.W. and Cartelli, D.M. (1983). Killing of intracellular *Leishmania donovani* by human mononuclear phagocytes. Evidence for oxygen-dependent and independent leishmanicidal activity. *J. Clin. Invest.* **72**, 32-44.

Murray, H.W. and Hariprashad, J. (1995). Interleukin 12 is effective treatment for an established

systemic intracellular infection experimental visceral leishmaniasis. *J. Exp. Med.* **181**, 387-391.

Murray, H.W. (1981). Susceptibility of *Leishmania* to oxygen intermediates and killing by normal macrophages. *J. Exp. Med.* **18**, 1302-1315.

Murray, H.W. (1982). Cell-mediated immune response to visceral leishmaniasis. II: Oxygen-dependent killing of intracellular *Leishmania donovani* amastigotes. *J. Exp. Med.* **153**, 1302-1315.

Murray, H.W. (1988). Interferon-gamma, the activated macrophage, and host defense against microbial challenge. *Ann. Intern. Med.* **108**, 595-608.

Murray, H.W. (1990). Effect of continuous administration of interferon-gamma in experimental visceral leishmaniasis. *J. Infect. Dis.* **161**, 992-994.

Murray, H.W. (1994). Cytokines in the treatment of leishmaniasis. Bailliere's *Clin. Infect. Dis.* **1**, 127-143.

Murray, H.W. (1994). Interferon and host animicrobial defense: current and future clinical applications. *Am. J. Med.* **97**, 459-67.

Murray, W., Hariprasad J. and Richard, E. (1993). Treatment of Experimental Visceral leishmaniasis in a `T' cell-Deficient host : Response to Amphotericin B and Pentamidine. *Antimicro. Agent and Chemo.* **37**, 1504.

Nag, J.K. and Ghosh, M.P. (1955). Kala-azar with pulmonary tuberculosis. *Ind. J. Med.* **25**, 138.

Naik, S.R., Rao, P.N., Datta, D.V., Mehta, S.K., Mahajan, R.C., Mehta, S. and Chuttani, P.N. (1979). Kala-azar in North Western India. A study of 24 patients. *Trans. Roy. Soc. trop. Med. Hyg.* **33**, 61.

Napier, L.E., Sengupta, P.C. and Sen, G.N. (1942). The treatment of kala-azar with stibamine. *Ind. Med. Gaz.* **77**, 321. Cited from Steck, E.A. (1974). In the leishmaniasis, *Prog. Drug. Res.* **18**, 283.

Neal, R.A., Croft, S.L. and Nelson, D,H, (1984). An *in vitro* system for determining the activity of compounds against the intracellular form of *Leishmania donovani*. *J. Antimicro. Chem.* **14**, 465.

Neal, R.A. (1968). The effect of antibiotics of the neomycin group on experimental cutaneous leishmaniasis. *Ann. Trop. Med. Parasitol.* **62**, 54-62.

Ng, K., Handman, E., Bacic, A. (1996). Biosynthesis of lipophosphoglycan from *L. major*: Solubilization and characterization of a (β1–>3) galactosyl transferase. *Biochem. J.* **317**, 247-256.

Nishizuka, J. (1984). The role of protein kinase c in cell surface signal transduction and tumor promotion. *Nature* **38**, 631.

Nishizuka, Y. (1986). Studies and perspectives of protein Kinase C. *Science* **233**, 305-312.

North, R.J. Neubauer, R.H., Huang, J.H., Newton, R.C., Loveless, S.E. (1988). Interleukin 1-induced T cell mediated regression of immunogenic murine tumors. Requirement for an adequate level of already acquired host concomitant immunity. *J. Exp. Med.* **168**, 2031-2043.

Olivier, M., and Tanner, C.E. (1989). The effect of cyclosporin A in murine visceral leishmaniasis. *Trop. Med. Parasitol.* **40**, 32-38.

Olliaro, P.L. and Bryceson, A.D.M. (1993). Practical progress and new drugs for changing patterns of leishmaniasis. *Parasitol. Today* **9**, 323-328.

Ookley, B.R., Kirsch, D.R., and Morris, N.R. (1980). A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels. *Anal. Biochem.* **105**, 361-363.

Opat, A. Ng, K., Currie, G., Handman, E., Bacic, A. (1996) Characterization of lipophosphoglycan from a Ricin-resistant mutant of *L. major*. *Glycobiol.* **6**, 387-398.

Orlandi, P.A. and Turco, S.J. (1987). Structure of lipid moiety of *Leishmania donovani* Lipophosphoglycan. *J. Biol. Chem.* **262**, 10384-10391.

Osborn, A.H., Kelleher, M., Handman, E. Goding, J.W. (1994). Anti-galactosyl antibodies that react with unmodified agarose: a potential source of artifacts in immunoaffinity chromatography. *Anal. Biochem.* **217**, 181-184.

Palatnik, C.B., Previato, J.O., Gorin, P.A.J. and Previato, M. (1985). Partial chemical characterization of the carbohydrate moieties in *Leishmania adleri* glycoconjugates. *Mol. Biochem. Parasitol.* **14**, 41-54.

Palmer, R.M.J., Ferrige, A.G. and Moncada, S. (1987). Nitric oxide release accounts for the biological activity of endothelium derived relaxing factor. *Nature* **327**, 524-526.

Pan, A.A. (1984). *Leishmania mexicana* serial cultivation of intracellular stages in a cell-free medium. *Exp. Parasitol.*,**58**, 72-80.

Panaro, M.A., Panunzio, M., Jirillo, E., Maranji, A. and Brandonisio, O. (1995). Parasite escape mechanisms. The role of *Leishmania* lipophosphoglycan on the human phagocyte functions a review. *Immunopharm. Immunotoxico.* **17**, 595-605.

Paul, E.W. (1993). Infectious diseases and the immune system. *Scientific American* **269**, 57-63.

Pearson, *et al.* (1996). Clinical spectrum of leishmaniasis. *Clin. Infect. Dis.* **220**, 1-13.

Pearson, R.D., Harcus, J.L., Roberts, D., Donowitz, G.R. (1983). Differential survival of *Leishmania donovani* amastigotes in human monocytes. *J. Immunol.* **131**, 1994-1999.

Pearson, R.D., Harcusk, J.L., Symes, P.H., Romito, R. and Donowitz, G.R. (1982). Failure of the phagocytic oxidative response to protect human monocyte-derived macrophages from infection by *Leishmania donovani. J. Immunol.* **129**, 1282-1286.

Perez-Soler, R., Lopez-Berestein, G., Johns, M., Wright, K., Kasi, L. (1985). Distribution of radiolabelled multilamellar liposomes injected intralymphatically and subcutaneously. *Int. J. Nuc. Med.* **12**, 261-266.

Peters, W., Bryceson, A., Evans, D.A., Neal, R.A., Kaye, P., Blackwell, J., Killick-Kendrick, R., and Leiw, F.Y. (1990). *Leishmania* infecting man and wild animals in Saudi Arabia and the influence of prior infection with *Leishmania arabica* on challenge with *L. major* on man. *Trans. Roy. Soc. Trop. Med. Hyg.* **84**, 681-684.

Peters, (1983). Chemotherapy of leishmaniasis-present status problems and prospects. In chemotherapy of *Leishmaniasis* present status problems and prospects. In Chemotherapy and immunology in the control of Malaria, Filariasis and *Leishmaniasis.*Edited Nityanand and Sen, A.B. Tata Mcgraw Hill Publishing Ltd., 243.

Phillips, W.A., Croatto, M. and Hamilton, J.A. (1990). Priming the macrophage respiratory burst with IL-4 : enhancement with TNF-α but inhibition by IFN-γ.*Immunol.* **70**, 498-503.

Piarroux, R., Gambarelli, F., Dumon, H., Fontes, M., *et al* (1994). Natural *Leishmania* infection of *Lutzomyia* spp. in Peru. *Trans. Roy. Soc. Trop. Med. Hyg.* **88**, 161-164.

Pimenta, P.F.P., Da Silva, R.P., Sacks, D.L. and Da Silva, P.P. (1989). Cell surface nanoanatomy of *Leishmania major* as revealed by fracture flip. A surface meshwork of 44 nm fusiform filaments identifies infective developmental stage promastigotes.*Eur. J. Cell Biol.* **48**, 180-190.

Pimenta, P.E.P., Saraiva, E.M., Rowton, E., Modi, G.B., Garraway, L.A., Beverley, S.M., Turco, S.J. and Sacks, D.L., (1994). The vectorial competence of phlebotomine sandflies for different species of *Leishmania* is controlled by structrual polymorphisms in the surface lipophosphoglycan. *Proc. Natl. Acad. Sci.* USA, **91**, 9155-9159.

Pimenta, P.E.P., Saraiva, E.M.B., Sacks, D.L. (1991). The comparative fine structure and surface glycoconjugate expression of three life stages of *Leishmania major.Exp. Parasitol.* **72**, 191-204.

Pimenta, P.F.P., Turco, S.J., McConville, M.J., Lawyer, P.G., Perkins, P.V. and Sacks, D.L. (1992). Stage specific adhesion of *Leishmania* promastigotes to the sandfly midgut. *Science.* **256**, 1812-1815.

Prasad, R., Tola, M., Bhattacharya, S., Sharma, M.P. and Bhattacharya, A. (1992). Recognition of *Entamoeba histolytica* lipophosphoglycan by strain-specific monoclonal antibody and human immune sera. *Mol. Biochem. Parasitol.* **56**, 279-288.

Preston, P.M. and Dumonde, D.C. (1976). Immunology of clinical and experimental leishmaniasis.

In "Immunology of Parasitic Infections" (S. Cohen and E.H. Sadun, eds.) 167-202. Blackwell Scientific Publications. Oxford.

Pritchard, D.G., Volanakis, J.E., Slutzky, G.M. and Greenblatt, C.L. (1985). C-reactive protein binds leishmanial excreted factors. *Proc. Soc. Exp. Biol. Med.* **178**, 500-503.

Proudfoot, L., O'Donnell, C.A. and Liew, F.Y. (1995). Glycoinositolphospholipids of *Leishmania major* inhibit nitric oxide synthesis and reduce leishmanicidal activity in murine macrophages. *Eur. J. Immunol.* **25**, 745-750.

Proudfoot, L., Schneider, P., Ferguson, M.A.J. and McConville, M.J. (1995). Biosynthesis of the glycolipid anchor of lipophosphoglycan and the structurally related glycoinositol phospholipids from *Leishmania major*. *Biochem. J.* **308**, 45-55.

Puentes, S.M., da Silva R.P., Sacks, D.L., Hammer, C.H., Joiner, K.A. (1990). Serum resistance of metacyclic stage *Leishmania major* promastigotes is due to release of C5b-9. *J. Immunol.* **145**, 4311-4316.

Puentes, S.M., Dwyer, D.M., Bates, P.A., Joiner, K.A. (1989). Binding and release of C3 from *Leishmania donovani* Promastigotes during incubation in normal human serum. *J. Immunol.* **143**, 3743-3749.

Puentes, S.M., Sacks, D.L., da Silva, R.P., Joiner, K.A. (1988). complement binding by two developmental stages of *Leishmania major* promastigotes varying in expression of a surface lipophosphoglycan. *J. Exp. Med.* **167**, 887-902.

Qiao, Z., Miles, M.A. & Wilson, S.M. (1995). Detection of parasites of the *Leishmania donovani* complex by a polymerase chain reaction solution hybridization enzyme linked immuno assay. *Parasitol.* **110**, 269-275.

Rachamim, N. and Jaffe, C.L. (1993). Pure protein from *L. donovani* protects mice against both cutaneous and visceral leishmaniasis. *J. Immunol.* **150**, 2322-2331.

Radzioch, D., Blottazzi, B., Varesio, L., (1987). Augmentation of C-fos mRNA expression by activators of protein kinase-C in fresh terminally differentiated resting macrophages. *Mol. Cell Biol.* **7**, 595-599.

Ramamoorthy, R., Donelson, J.E., Paetz, E.E., Maybodi, M., Roberts, S.C. and Wilson, M.E. (1992). Three distinct RNAs for the surface protease gp63 are differentially expressed during development of *Leishmania donovani Chagasi* promastigotes to an infectious form. *J. Biol. Chem.* **267**, 1888-1895.

Raz, A., Bucana, C., Fogler, W., Poste, G., Fidler, I. (1981). Biochemical, morphological and ultrastructural studies on the uptake of liposomes by murine macrophages. *Can. Res.* **41**, 487-494.

Rees, P.N., Kagar, P.A., Ogade, T., Eeftinck, J.K., Schattenkert. (1985). The treatment of kala-azar a review with comment drawn Experience in Kenya. *Trop. George Med.* **37**, 37. Cited from *Trop. Dis. Bull.* **83** (1986) 1148.

Rees, P.N. and Kagar, P.A. (1987). Visceral leishmaniasis and post kala-azar dermal leishmaniasis. In the leishmaniasis in Biology and Medicine volume 1. Clinical aspects and control. Edited by W. Peter and R. Killick-Kendrick Academic Press, U.K. 563.

Reiner, N.E. and Malemud, C.J. (1985). Arachidonic acid metabolism by murine peritoneal macrophages infected with *Leishmania donovani: in vitro* evidence for parasite-induced alterations in cycloxygenase and lipoxygenase pathways. *J. Immunol.* **134**, 556-563.

Reiner, N.E., Ng. W. and McMaster, W.R. (1987). Parasite accessory cell interactions in murine leishmaniasis II. *Leishmania donovani* suppresses macrophage expression of class I and class II major histocompatibility complex gene products. *J. Immun.* **138**, 1926-1932.

Reiner, N.E., Kazura, J.W. (1982). Oxidant mediated damage of *Leishmania donovani* promastigotes. *Infect. Immun.* **36**, 1023-1027.

Reiner, N.E. and Finke, J.H. (1983). Interleukin 2 deficiency in murine leishmaniasis and its relationship to depressed spleen cell respnse to phytohemagglutinin. *J. Immunol.* **131**, 1487-1491.

Reiner, N.E. and Malemud, C.J. (1984). Arachidonic acid metabolism in murine leishmaniasis (donovani): *ex vivo* evidence for increased cycloxygenase and 5-lipoxygenase activity in spleen cells. *Cell. Immunol.* **88**, 501-510.

Reiner, N.E. (1987). Parasite accessory cell interactions in murine leishmaniasis I. Evasion and stimulus-dependent suppression of the macrophage interleukin-I response by *Leishmania donovani*, *J. Immunol.* **138**, 1919-1925.

Reiner, S.H., Zheng, S., Wang, Z.E., Stowring, L. and Locksley, R.M. (1994). *Leishmania* promastigotes evade interleukin 12 (IL-12) induction by macrophages and stimulate a broad range of cytokines from CD4+ T-cells during initiation of infection. *J. Exp. Med.* **179**, 447-456.

Reiner, S.L., Wong, Z.E., Hatam, F., Scott, P. and Locksley, R.M. (1993). Th1 and Th2 cell antigen receptors in experimental leishmaniasis. *Science.*, **259**, 1457-1460.

Reiner, S.R. and Locksley, R.M. (1995). The regulation of immunity to *Leishmania major*. *Annu. Rev. Immunol.* **13**, 151-177.

Rezai, H.R., Behbehani, A.B., Gettner, S. and Ardehali, S. (1988). Effect of levamisole on the course of experimental leishmaniasis in guinea pigs and mice haematological and immunological findings. *Ann. Trop. Med. Parasitol.* **82**, 243-249.

Rezai, H.R., Behfrouz, N., Amiphakimi, G.H., Kohanter, J. (1977). Immunofluorescence and

counter immunoelectrophoresis in diagnosis of kala-azar. *Trans. Roy. Soc. Trop. Med. Hyg.* **71**, 149-151.

Rizvi, F.S., Quaissi, M.A., Marty, B., Santoro, F. and Capron, A. (1988). The major surface protein of *Leishmania* promastigotes is a filaronectin like molecule. *Eur. J. Immunol.* **18**, 473-478.

Roach, T.I.A., Kidereln, A.F. and Blackwell, J.M. (1991). Role of inorganic nitrogen oxides and tumour necrosis factor alpha in killing *Leishmania donovani* amastigotes in gamma interferon lipopolysaccharide activated macrophages from Lsh^s and Lsh^r congenic mouse strains. *Infect. Immuno.* **59**, 3935-3944.

Roditi, I., Schwarz, H., Pearson, T.W., Beecroft, R.P., Liu, M.K., Richardson, J.P., buhring, H.J., Pleiss, J., bulow, R., Williams, R.O. and Overath, P. (1989). Procyclin gene expression and loss of the variant surface glycoprotein during differentiation of *Trypanosoma brucei*. *J. Cell. Biol.* **108**, 737-746.

Rodriguez, N., Guzman, B., Rodas, A., *et al* (1994). Diagnosis of cutaneous leishmaniasis and species discrimination of parasites by PCR and hybridisation. *J. Clin. Microbiol.* **32**, 2246-2252.

Rosen, G., Londner, M.V., Sevlever, D., Greenblatt, C.L. (1988). *Leishmania major*: glycolipid antigens recognized by immune human sera. *Mol.Biochem. Parasitol.* **27**, 93-100.

Rosen, G., Pahlsson, P., Londner, M.V., Westerman, M.E., Nilsson, B. (1989). Structural analysis of glycosyl-phosphatidylinositol antigens of *Leishmania major*. *J. Biol. Chem.* **264**, 10457-10463.

Russell, D.G., Alexander, J. (1988). Effective immunization against cutaneous leishmaiasis with defined membrane antigens reconstituted into liposomes. *J. Immunol.* **140**, 1274-1279.

Russell, D.G., Talamas-Rosana, P. (1989). *Leishmania* and macrophage: A marriage of inconvenience. *Immunol.Today* **10**, 328-333.

Russell, D.G., Wilhelm, H. (1986). The involvement of the major surface glycoprotein (gp63) of *Leishmania* promastigotes in attachment to macrophages. *J. Immunol.* **136**, 2613-2620.

Russell, D.G. and Wilhelm, H. (1986). The involvement of the major surface glycoprotein (gp63) of *leishmania* promastigotes in attachment to macrophages. *J. Immunol.* **136**, 2613-2620.

Russell, D.G. Wright, S.D., (1988). Complement receptor type 3 (CR3) binds to an Arg-Gly-Asp containing region of the major surface glycoprotein gp63, of *Leishmania* promastigotes. *J. Exp. Med.* **168**, 279-292.

Russell, D.G. (1987). The macrophage attachement glycoprotein gp63 is the predominant C-3-acceptor site on *Leishmania mexicana* promastigotes. *Eur. J. Biochem.* **164**, 213-221.

Russell, D.G. (1990). *Leishmania* and the macrophage. *Immunol. Today* **11**, 74-75.

Russo, D.M., Burns, Jr. J.M., Carvalho, E.M., *et al* (1991). Human T-cell responses to rgp63, a surface antigen of *Leishmania*. *J. Immunol.* **147**, 3575-3580.

Russo, D.M., Reed, S.G. (1991). Human T- cell responses to gp63, a surface antigen of *Leishmania*. *J. Immunol.* **147**, 3575-3580.

Russo, D.M., Turco, S.J., Burns, J.M., Reed, S.G. (1992). Stimulation of human T-Lymphorytes by *Leishmania* lipophosphoglycan associated proteins. *J. Immunol.* **148**, 202-207.

Ryan, K.A., Garraway, L.A., Descoteaux, A., Turco, S.J. and Beverley, S.M. (1993b). Isolation of virulence genes directing surface GPI synthesis in protozoan parasites. *Proc. Natl. Acad. Sci.* USA, **90**, 8609-8613.

Sacks, D.L., Brodin, T., Turco, S.J. (1990). Developmental modification of the lipophosphoglycan from *Leishmania major* promastigotes during metacyclogenesis. *Mol. Biochem. Parasitol*, **42**, 225-234.

Sacks, D.L., da Silva, R.P. (1987). The generation of infective stage *Leishmania major* promastigotes is associated with the cell surface expression and a release of a developmentally regulated glycolipid. *J. Immunol.* **139**, 3099-3106.

Sacks, D.L., hieny, S., Sher, A. (1985). Identification of cell surface carbohydrate and antigenic changes between non-infective and infective developmental stages of *Leishmania majro* promastigotes. *J. Immunol.* **135**, 564-569.

Sacks, D.L., Latahal, S., Shrivastava, S.N., Blackwell, J.M. and Neva, F.A. (1987). An analysis of T-cell responsiveness in Indian kala-azar. *J. Immunol.* **138**, 908-913.

Sacks, D.L., Pimenta, P.F.P., McConville, M.J., Schneider, P. and Turco, S.J. (1995). Stage specific Binding of *Leishmania donovani* to the sandfly vector midgut is regulated by conformational changes in the abundant surface lipophosphoglycan. *J. Exp. Med.* **181**, 685-697

Sacks, D.L., Saraiva, E.M., Rowton, E., Turco, S.J. and Pimenta, P.F. (1994). The role of the lipophosphoglycan of *Leishmania* in vector competence. *Parasitol.* **108**, S55-S62.

Sacks, D.L. and Perkins, P.V. (1984). Identification of an infective stage of *Leishmania* promastigotes. *Science*. **233**, 1417-1419.

Sacks, D.L. and Perkins, P.V. (1985). Development of infective stage *leishmania* promastigotes within phlebotomine sandflies. *Am. J. Trop. Med. Hyg.* **34**, 456-459.

Sacks, D.L. (1989). Metacyclogenesis in *Leishmania* Promastigortes. *Exp. Parasitol.* **69**, 100-103.

Saha, A.K., Mukherjee, T., and Bhaduri A.P. (1986). Mechanism of action of Amphotericin B on *Leishmanai donovani* Promatigotes *Mol. Biochem. Parasitol.* **19**, 195-200.

Salle, A.J., Schmidt, C.L.A. (1928). The metabolism of *L. tropica*. *J. Infec. Dis.* **43**, 378-384.

Saraiva, E.M.B., Pimenta, P.F.P., Brodin, T.N., Rowton, E., Modi, G.B. and Sacks, D.L. (1995) Changes in lipophosphoglycan and gene expression associated with the development of *Leishmania major* in *Phlebotomus papatasi*. *Parasitol.* **111**, 275-287.

Satoskar, A., Bluethmann, H. and Alexander, J. (1995). Disruption of the murine interleukin-4 gene inhibits disease progression during *Leishmania mexicana* infection but does not increase control of *Leishmania donovani* infection. *Infect. Immun.* **63**, 4894-4899.

Schlein, Y., Schur, L.F. and Jacobson, R.L. (1990). Released glycoconjugate of indigenous *Leishmania major* enhance survival of a foreign *L. major* in *Phlebotomus papatasi*. *Trans. Roy. Soc. Trop. Med. Hyg.* **84**, 353-355.

Schlein, Y. and Jacobson, R.L. (1994). Mortality of *Leishmania major* in *Phlebotomus papatasi* by plant feeding of the sandflies. *Am. J. Trop. Med. Hyg.* **50**, 20-27.

Schneider, P., Ferguson, M.A.J., McConville, M.J., Mehlert, A., Homans, S.W., *et al.* (1990). Structure of the glycosyl-phosphatidylinositol membrane anchor of the *Leishmania major* promastigote surface protease, *J. Biol. Chem.* **265**, 16955-16964.

Schneider, P., Rosat, J.P., Bouvier, J., Louis, J. and Bordier, C. (1992). *Leishmania major* : Differential regulation of the surface melalloprotease in amastigotes and promastigote stages. *Exp. Parasitol.* **75**, 196-206.

Schneider, P., Rosat, J.P., Ransijn, A., Ferguson, M.A.J. and McConville, M.J. (1993). Characterization of glycoinositol phospholipids (GIPLS) in the amastigote stage of the protozoan parasite *Leishmania major*. *Biochem. J.* **295**, 555.

Schnur, L.F., Zuckerman, A. and Greenblatt, C.L. (1972). Leishmanial serotypes as distinguished by the gel diffusion of factors excreted *in vitro* and *in vivo*. *Isr. J. Med. Sci.* **8**, 932-942.

Schottelius, J. (1982). Lectin binding strain specific carbohydrates on the cell surfaces of *Leishmania* strains from the Old World. Zeitschrift fur Parasitenkunde. **66**, 237-247.

Schottelius, J. (1982). Lectin typing of *Leishmania* strains from the new and old world, P-531-541. In T.C. Bog. Hansen (ed). Lectins biology Biochemistry clinical biochemistry. Vol. II. Walter de Gruyter and Co. Berlin.

Scott, P.A., Natovitz, P., Coffman, R.L., Pearce, E. and Sher, A. (1988). Immunoregulation of cutaneous leishmaniasis T cell lines that transfer protective immunity or exacerbation belong to different T helper subsets and respond to distinct parasite antigens. *J. Exp. Med.* **168**, 1675-1684.

Scott, P. (1993). IL-12: initiation cytokine for cell mediated immunity. *Science*. **260**, 496.

Scott, P. (1993). Selection differentiation of $CD4^+$ T helper cell subsets. *Curr. Opin. Immunol.* **5**, 391-397.

Scott, P.A., James, S.L. and Sher, A. (1985). The respiratory burst is not required for killing intracellular and extracellular parasites by a lymphokine activated cell line. *Eur. J. Immunol.* **15**, 553-561.

Scott, P.A., Pearce, E., Cheever, A.W., Coffmann, R.L. and Sher, A. (1989). Role of cytokines and $CD4^+$ T cell subsets in the regulation of parasite immunity and disease. *Immunol. Rev.* **112**, 161-182.

Scott, P.A. and Farrell, J.P. (1981). Experimental cutaneous leishmaniasis I: Nonspecific immunodepression in BALB/c mice infected with *Leishmania tropica*. *J. Immunol.* **127**, 2395-2400.

Seaman, *et al.* (1995). Liposomal Amphotericin B (AmBisome) in the treatment of complecated Kala-azar under field conditions. *Clin. Infect. Dis.* **21**, 188-193.

Sen Gupta, P.C. and Chakraborty, N.K. (1956). The liver in kala-azar. *Ann. Trop. Med. Parasitol.* **50(3)**, 252.

Sen Gupta, P.C. (1944). *Ind. Med. Gazz.* **79**, 50. Cited from steck, E.A., in the *Leishmaniasis. Prog. Drug. Res.* **18**, 283.

Sen Gupta, P.C. (1950). *Ind. Med. Gazz.* **85**, 547. Cited from Steck, E.A. (1974). In the *Leishmaniasis. Prog. Drug. Res.* **18**, 263.

Shapira, M., McEwen, J.G., Jaffe, C.L. (1988). Temperature effects on molecular processes which lead to stage differentiation of *Leishmania*. *EMBO J.* **7**, 2895-2901.

Shaw, J.J. and Lainson, R. (1975). Leishmaniasis in Brazil. X. Some observations on intradermal reactions to different trypanosomatid antigens of patients suffering from cutaneous and mucocutaneous leishmaniasis. *Trans. Roy. Soc. Trop. Med. Hyg.* **69**, 323-335.

Shaw, J.J. and Lainson, R. (1974). An immediate reaction to leishmanial antigen in human cutaneous leishmaniasis. *Trans. Roy. Soc. Trop. Med. Hyg.* **68**, 168-169.

Shaym, S., Rosenkaimer, F., Lesser, M.L. and Murray, H.W. (1995). Immunochemotherapy for a systemic intracellular infection: accelerated response using interferon-gamma in visceral leishmaniasis. *J. Infect. Dis.* **171**, 992-996.

Sheiky, Y.A.W., Benson, D.R., Elwasila, M., Badaro, R., *et al.*(1994). Shared antigens between *Leishmania* and *Trypanosoma cruzi*: characterization of the *Leishmania chagasi* acidic ribosomal protein PO. *Infect. Immun.* **62**, 1643-1651.

Sher, A. and Coffman, L.R. (1992). Regulation of immunity to parasite by T-cell and T-cell derived cytokines. *Ann. Rev. Immunol.* **10**, 385-409.

Simpson, L. (1968). Behavior of the kinetoplast of *Leishmania tarentolae* upon cell rupture. *J. Protozol.* **15**, 132-136.

Singh, B.N. and Beach, D.H., Lindmark, D.G. and Costello, C.E. (1994). Identification of lipid moiety and further characterization of the novel lipophosphoglycan-like glycoconjugate of *Trichomonas vaginalis* and *Trichomonas foetus*. *Arch. Biochem. Biophys.*, **309**, 273-280.

Singh, B.N. (1993). Lipophosphoglycan like glycoconjugate of *Tritrichomonas foetus* and *Trichomonas vaginalis*. *Mol. Biochem. Parasitol.* **57**, 281-294.

Singh, B.N. (1994). The existance of lipophosphoglycan-like molecules in *Trichomonads*. *Parasitol. Today*, **10**, 152.

Singha, U.K. (1990). Studies on chemotherapeutic evaluation of natural products and for synthetic compounds for anti-leishmanial action against *L. donovani* in golden hamsters, Ph.D. thesis, Kanpur University, India.

Sivaramakrishnamaiah, K. and Ramanathan, R. (1967). Studies on the effect of climate on leishmaniasis in India. *Ind. J. Med. Res.* **55**, 1159.

Skov, C.B. and Twohy, D.W. (1974a). Cellular immunity to *Leishmania donovani*. I: The effect of T cell depletion on resistance to *L. donovani* in mice. *J. Immunol.* **113**, 2004-2011.

Skov, C.B. and Twohy, D.W. (1974b). cellular immunity to *Leishmania donovani* II : Evidence for synergy between thymocytes and lymph node cells in reconstitution of acquired resistance to *L. donovani* in mice. *J. Immunol.* **113**, 2012-2019.

Slutzky, G.M., and Greenblatt, C.L. (1982). Identification of galactose as the immunodominant sugar of leishmanial excreted factor and subsequent labelling with galactose oxidase and sodium boro [^{3}H]hydrate. *Infect. Immun.* **37**, 10-14.

Slutzky, G.M., El-on, J. and Greenblatt, C.L. (1979). *Leishmania* excreted factor: protein bound and free forms from promastigotes cultures of *Leishmania Tropica* and *L. donovani*. *Infect. Immun.* **26**, 916-924.

Smith, D.F. and Rangarajan, D. (1995). Cell surface components of *Leishmania*: identification of a noevl lectin. *Glycobiol.* **5**, 161-166.

Smith, L.E. Rodrigues, M. and Russell, D.G. (1991). The interaction between CD8+ cytotoxic T-cells and *Leishmania-infected* macrophages. *J. Exp. Med.* **174**, 499-505.

Smith, S.W. and Lester, R.L. (1974). Inositol phosphorylceramide, a novel substance and the chief member of a major group of yeast sphingolipids containing a single inositol phosphate. *J. Biol. Chem.* **249**, 3395-3405.

Snaper, I. (1952). American mucocutaneous leishmaniasis, successfully treated with hydroxystilbamidine. *Am. J. Med.* **13**, 655-664.

Sogin, M.L., Gunderson, J.H., Elwood, H.J., Alonso, R.A. and Pealtie, D.A. (1989). Phylogenetic meaning of the kingdom concept: An unusual ribosomal RNA from the *Giardia lamblia. Sci.* **243**, 75-77.

Solbach, W., Moll, H. and rollinghoff, M. (1991). Lymphocytes play the music but the macrophage plays the tune. *Immunol. Today* **12**, 4-6.

Solbach, W. and Laskay, T. (1995) *Leishmania major* infection. *Parasitol,Today*, **70**, 394-397.

Southgate, B.A. and Manson-Bahr, P.E.C. (1967). Studies in the epidemiology of East American leishmaniasis. The significance of the positive leishmanin test. *J. Trop. Med. Hyg.* **70**, 29-33.

Squires K.E., Rosenkaimer, F., Sherwood, J.A., Were, J.B.O., Murray, H.W. (1993). Immunochemotherapy for visceral leishmaniasis: a controlled pilot trial of antimony vs. antimony plus interferon. *Am. J. Trop. Med. Hyg.* **48**, 666-669.

Srivastava, G., Tola, M.A., Bhattacharya, S. and Bhattacharya, A. (1995). Lipophosphoglycan is present in distinctly different form in different *Entamoeba histolytica* strains and absent in *Entamoeba moshkovsii* and *Entamoeba invadens. J. Euk. Microbiol.* **42**, 617-622.

Stanley, S.L. Jr., Huizenga, H. and Li, E. (1992). Isolation and partial characterization of a surface glycoconjugate of *Entamoeba histolytica. Mol. Biochem. Parasitol.* **50**, 127-138.

Stauber, L.A., Jackson, G.J., Herman, R. and Singer, I. (1970). *Leishmania* in "immunity to parasitic animals". **2**, 739-765, Applenton, New York.

Steck, E.A. (1974). The *Leishmaniasis,Prog. Drug. Res.* **18**, 289.

Stefani, M.M.A., Muller, I. and Louis, J.A. (1994). *Leishmania major* specific $CD4^+$ T cells are inducers and targets of nitric oxide produced by parasitized macrophages. *Eur. J. Immunol.* **24**, 746-752.

Straus, A.H., Levery, S.B., Jasiulionist, M.G., Salyan, M.E.K., Steele, S.J., Travassos, L.R., Hakomori, S. and Takahasbi, H.K. (1993). Stage-specific glycosphingolipids from amastigote forms of *Leishmania amazonensis. J. Biol. Chem.* **268**, 13723-13730.

Sundar, S., Rosenkaimer, F., Murray, H.W. (1994). Successful treatment of refractory visceral leishmaniasis in India using antimony plus interferon-γ. *J. Infect. Dis.* **170**, 659-662.

Sunder, S., Murray, H.W. (1995). Effect of treatment with interferon-γ alone in visceral leishmaniasis. *J. Infect. Dis.* **172**, 1627-1629.

Sunder S., Thakur, B., Mishra, A., Rosenkaimer, F., Murray, H.W. (1993). Accelerated response

using interferon plus antimony in visceral leishmaniasis in India. *Clin. Infect. Dis.* **17**, 545.

Sypek, J.P., Chung, C.L., Mayor, S.E. *et al.* (1993). Resolution of cutaneous leishmaniasis : Interleukin 12 initiates a protective T-helper type 1 immune response. *J. Exp. Med.* **177**, 1797-1802.

Tait, A. and Sacks, D.L. (1988). The cell biology of parasite invasion and survival. *Parasitol. Today* **4**, 228-234.

Talamas-Rohana, P., Wright, S.D., Lennartz, M.R., Russell, D.G. (1990). Lipophosphoglycan from *Leishmania mexicana* promastigotes binds to members of the CR3, P150,95 and LFA-1 family of leukocytes integrins. *J. Immunol.* **144**, 4817-4824.

TDR News Letter (1990). Antimonials: Large scale failure in leishmaniasis alarming. No. 34 December.

Teinia, A.K. and Soteriadou, K.P. (1991). Substrate dependent pH optima of gp63 purified from seven strains of *Leishmania. Mol. Biochem. Parasitol.* **47**, 83-90.

Thakur, C.P., Pandey, A.K., Sinha, G.P., Roy, S. Behbehani, K., Olliaro, P. (1996). Comparasion of three treatment regimens with liposomal amphotericin B (AmBisome) for visceral leishmaniasis in India: A randomized dose finding study. *Trans. Roy. Soc. Trop. Med. Hyg.* **90**,319-321.

Thakur, C.P., Kumar, K., Sinha, P.K., Mishra, B.N. and Pandey, A.K. (1988). Rationalization of regimens of treatment of kala-azar with sodium stibogluconate in India a randomised study. *Brit. Med. J.* **296**, 1557.

Thakur, C.P. and Kumar, K. (1990). Efficacy of prolonged therapy with stibogluconate in kala-azar (including post kala-azar dermal leishmaniasis). *Trans. Roy. Soc. Trop. Med. Hyg.* **78**, 391.

Thakur, C.P. (1984). Epidemiological, Clinical and therapeutic features of Bihar kala-azar (including PKDL). *Trans. Roy. Soc. Trop. Med. Hyg.* **78(3)**, 371.

Thakur, C.P. (1990). Prolonged with stibogluconate in post kala-azar-dermal leishmaniasis. *Ind. J. Res.* **91**, 144.

Thomas, J.R., McConville, M.J., Thomas-Oates, J.E., Homans, S.W., Ferguson, M.A.J., *et al.* (1992). Refine structure of the lipophosphoglycan of *Leishmania donovani J. Biol. Chem.* **267**, 6829-6833.

Titus, R.G., Ceredig, R., Cerottini, J.C. and Louis, J.A. (1985). Therapeutic effect of anti-L3T4 monoclonal antibody GK 1.5 on cutaneous leishmaniasis in genetically susceptible BALB/c mice. *J. Immunol.* **135**, 2108-2114.

Tolson, D.L, Turco, S.J., Pearson, T.W. (1990). Expression of a repeating phosphorylated disaccharide lipophosphoglycan epitope on the surface of macrophages infected with *Leishmania*

donovani. Infect. Immun. 58, 3500-3507.

Tolson, D.L., Turco, S.J., Beecroft, R.P., Pearson, T.W., (1989). Immunochemical and cell surface arrangement of the *Leishmania donovani* lipophosphoglycan determined using monoclonal antibodies. *Mol. Biochem. Parasitol.* 35, 109-118.

Towbin, H., Rosenfelder, G., Wieslander, J., Avila, J.L., Rojas, M., *et al.* (1987). Circulating antibodies to mouse laminin in cha gas disease, American cutaneous leishmaniasis and normal individuals recognize terminal galactosyl (α1,3) galactose epitopes. *J. Exp. Med.* 166, 419-432.

Tremblay, M., Olivier, M., Berneir, R. (1996). *Leishmania* and pathogenesis of HIV-infection. Parasitol. Today 12, 257-260.

Trotter, E.R., Peters, W. and Robinson, B.L. (1980a). The experimental chemotherapy of leishmaniasis IV, the development of a rodent model for V.I. *Ann. Trop. Med. Parasitol.* 74, 127-138.

Trotter, E.R., Peters, W. and Robinson, B.L. (1980b). The experimental chemotherapy of leishmaniasis IV, the development of a rodent model for cutaneous infection with *L. major* and *L. mexicana amazonesis. Ann. Trop. Med. Parasitol.* 74, 298-319.

Tumang, M., Keogh, C., Moldawer, L., Helfgott, D., Hariprashad, J. and Murry, H.W. (1994).Role and effect of TNF_{α} in experimental visceral leishmaniasis. *J. Immunol.* 153, 768-775.

Turco, S.J., Orlandi, P.A., Jr. Homans, S.W., Ferguson, M.A.J., Dwek, R.A., *et al.*, (1989). Structure of the phosphosaccharide-inositol core of the *L. donovani* lipophosphoglycan. *J. Biol. Chem.* 264, 6711-6715.

Turco, S.J., Orlandi, P.A., Shepherd, S.D., Homans, S.W., Ferguson, M.A.J., Dwek, R.A. and Rademacher, T.W. *et al.* (1987). Structure of major carbohydrate fragment of *Leishmania donovani* lipophosphoglycan. *Biochem.* 26, 6233-6238.

Turco, S.J., Wilkerson, M.A. and Clawson, D.R. (1984). Expression of an unusual acidic glycoconjugate in *Leishmania donovani. J. Biol. Chem.* 259, 3883-3889.

Turco, S.J. and Descoteaux, A. (1992). The Lipophosphoglycan of *Leishmania* parasites. *Annu. Rev. Microbiol.*46, 65-94.

Turco, S.J. Sacks, D.L. (1991). Expression of a stage specific lipophosphoglycan in *L. major* amastigotes. *Mol. Biochem. Parasitol.* 45, 91-100.

Turco, S.J. (1988). Proposal for a function of the *Leishmania donovani lipophosphoglycan. Biochem. Soc. Trans.* 16, 259-261.

Turco, S.J. (1988). The lipophosphoglycan of *Leishmaia. Parasitol. Today*, 4, 255.

Turco, S.J. (1990). The leishmanial lipophosphoglycan: A multifunctional molecule. *Ex. Parasitol.* **70**, 241-245.

Turco, S.J. (1992). The lipophosphoglycan of *Leishmania. Subcell. Biochem.* **18**, 73-97.

Turk, J.L., Bryceson, A.D.M. (1971). Immunological phenomena in Leprosy and related diseases. *Adv. Immunol.* **13**, 209-266.

T.D.R. News Letter (1991). Published by UNDP/World Bank/WHO special programme for Research and training in Tropical Disease. No. **36**, June.

Unanue, E.R., Allen, P.M. (1987). The basis for the immuno regulatory role of macrophages and other accessory cells. *Sci.* **236**, 551-557.

Unanue, E.R. (1984). Antigen-presenting function of the macrophage. *Ann. Rev. Immunol.* **2**, 395-428.

Van der Ploeg, LHT. (1985). Heat shock genes regulatory role for differentiation in parasitic protozoa. *Sci.* **228**, 443-446.

Vieira, L., Lavan, A., Dagger, F. Kabantchik, Z.I. (1994). The role of anieas in pH regulation of *Leishmania major* promastigotes. *J. Biol. Chem.* **269**, 16254.

Volf, P., (1994). Comparison of the haemagglutination activities in gut and lead extracts of various species and geographical populations of *Phlebotomins* sandflies. *Ann. Trop. Med. Parasitol.* **88**, 337-340.

Wali, J.P., Agarwal, P., Gupta, V., Saluja, S. and Singh, S. (1990). Ketoconazole in treatment of visceral leishmaniasis. *Lancet* **336** (8718), 810.

Wali, J.P., (1990). Ketoconazole in visceral liehsmaniasis. *Lancet* II, 1582-1583.

Walters, L.L. (1993). *Leishmania* differentiation in natural and unnatural sandfly hosts. *J. Euk. Microbiol.* **40**, 196-206.

Wasan, K.M., and Lopez-Berestein, G. (1995). The past, Present and future uses of liposomes in treating infectious diseases. *Immunopharmacol. and Immunotoxicol.* **17**, 1-15.

Wassef, M.K.T.B. Fioretti, B. and Dwyer, D.M. (1985). Lipid analysis of isolated surface membranes of *Leishmania donovani* promastigotes. *Lipids* **20**: 108-115.

Weaver, C.T. and Unanue, E.R. (1990). The co-stimulatory function of APC. *Immunol. Today* **11**, 49-53.

Wilson, M.D. and Pearson, R.D. (1984). Stage-specific variations in lectin binding to *Leishmania donovani*. *Infect. Immun.* **46**, 128-132.

Wilson, M.E., Pearson, R.D. (1986). Evidence that *Leishmania donovani* utilizes a mannose receptor on human mononuclear phagocytes to establish intracellular parasitism. *J. Immunol.* **136**, 4681-4688.

Wilson, M.E., Pearson, R.D. (1988). Role of CR3 and mannose receptors in the attachement and ingestin of *Leishmania donovani* by human mononuclear phagocytes. *Infect. Immun.* **56**, 363-369.

Wolff, B., and Gregoriadis, G. (1984). The use of monoclonal antibodies thy$_1$ IgG1 for the targetting of liposomes to AKR-cells *in vitro* and *in vivo*. *Biophys.Acta* **802**, 259-273

Wright, E.P. and El-Amin, E.R.M. (1989). *Leishmania* infection: surfaces and immunity *Biochem. Cell Biol.* **67**, 525-536.

Wright, S.D., Silverstein, S.C. (1983). Receptors for C3b and C3bi promote phagocytosis but not the release of toxic oxygen from human phagocytes. *J. Exp. Med.* **156**, 2016-2023.

Wyler, D.J., Sypek, J.P. and McDonald, J.A. (1985) *In vitro* parasite-monocyte interactions in human leishmaniasis: possible role of fibronectin in parasite attachment. *infect. Immun.* **49**, 305-311.

Yang, D.M., Fairweather, N., Button, L., *et al.*(1990). Oral *S. typhimurium* (AroA- vaccine expressing leishmanial surface protein (gp63) preferentially induced Th1 cells and protective immunity against leishmaniasis. *J. Immunol.*145, 2281-2285.

Zilberstein, D. and Shapira, M. (1994). The role of pH and temperature in the development of *Leishmania* parasite. *Annu. Rev. Microbiol.* **48**, 449-470.

Zilberstein, D. Blumenfeld, N. Liveanu V. Gepstein, A., Jaffe C.L. (1991). Growth at acidic pH induces an amastigote stage specfic protein in *Leishmania* promastigotes. *Mol. Biochem. Parasitol.* **45**, 175-78.

Zilberstein, D. (1991). Adaptation of *Leishmania* species to an acidic environment. In *biochem. Protozoa*343-358.

Zilberstein, D. (1993). Transport of nutrients and ions across membranes trypanosomatid parasites. *Adv. Parasitol.***32**, 261-91.

Zuckerman, A., Lainson, R. (1977). In "Parasitic protozoa", (J.P. Kreier, Ed.) **1**, 58-134, Academic Press, New York.

Zuckerman, A. (1975). Current status of the immunology of blood and tissue protozoa. **1**. *Leishmania. Exp. Parasitol.* **38**, 370-400.